UNIVERSITY OF ALBERTA

Development of the Indicator Amino Acid Oxidation Method for the Determination of the Metabolic Availability of Lysine in Feedstuffs in Broilers

by

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Abstract

The Indicator Amino Acid Oxidation (IAAO) method was developed for use in broilers for the determination of lysine availability in feedstuffs. The bicarbonate retention factor (BRF) for broilers was determined using a primed oral dose protocol with $Na^{14}CO_3$. A function that allows prediction of the BRF using body weight was developed. The appropriate priming and hourly oral dose of L-[1-^{14}C]phenylalanine was determined using time to plateau as the response. A ratio of 0.87 (prime to hourly oral dose) was found to be appropriate. The period of adaptation required to reach a new steady state of indicator oxidation after a change in dietary lysine content was determined to be two days for both increases and decreases in dietary lysine. The IAAO method was tested against the slope ratio method for its ability to determine the availability of lysine in peas, and similar values were obtained. This shows the IAAO method can be used to determine the availability of amino acids in feedstuffs.

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TABLE OF CONTENTS

CHAPTER 1: LITERATURE REVIEW 1

Background 1

Amino Acid Availability 3

Biological Value 3

Net Protein Utilization 5

Protein Efficiency Ratio 6

Net Protein Ratio 7

Nitrogen Balance 7

Protein Digestibility-Corrected Amino Acid Score 8

Digestibility 10

Slope Ratio Technique 12

Direct Oxidation 14

Indicator Amino Acid Oxidation 15

IAAO Procedures 16

Criticisms of the IAAO Procedure 18

IAAO Availability Method 22

Research Objectives 23

References 25

References 25

Tables and Figures 35

CHAPTER 2: BICARBONATE RETENTION AND BONE DENSITY 36

Introduction 36

MATERIALS AND Methods 38

Stock and Management 38

Experimental Design 38

Statistical Analysis 40

Results and Discussion 41

References 48

Tables and Figures 52

CHAPTER 3: ADAPTATION PERIOD AND PHENYLALANINE DOSE LEVELS 57

Introduction 57

Materials and Methods 61

Stock and management 61

Experimental Design 61

Statistical analysis 64

Results and Discussion 65

References 71

Tables and Figures 76

CHAPTER 4: SLOPE RATIO AND IAAO AVAILABILITY 81

Introduction 81

Materials and Methods 82

Statistical Analysis 87

Results and Discussion 88

References 94

CHAPTER 5: CONCLUSION 114

References 118

LIST OF TABLES

Table 2.1. Ingredient and calculated nutrient composition of commercial type starter (0-21 d) and grower (22-42 d) diets 52

Table 2.2. Weekly body weight, and bicarbonate retention in broilers age 0-42 d, fed a commercial type diet free choice[1,2] 53

Table 2.3. Body weight, bone density, and bone area of the femur in broiler chicks from 0 to 42 days of age (mean ± standard error) 54

Table 2.4. Correlations between body weight, age, bicarbonate retention and bone traits of broilers[1] 55

Table 3.1. Ingredients and calculated composition of purified, pelleted grower diets (Experiment 2) 76

Table 3.2. Priming dose and hourly dose of ^{14}C-phe, and resulting time to plateau of birds fed a commercial diet 77

Table 3.3. Mean % ^{14}C-phe dose oxidized and SEM for points on different oxidation days in Experiments 1 and 2 78

Table 4.1. Dietary ingredients and calculated nutrient composition of diets for the slope ratio experiment (Experiment 1) determining the availability of lysine in a sample of peas. 98

Table 4.2: Dietary amino acid composition of diets for the slope ratio experiment (Experiment 1) determining the availability of lysine in a sample of peas, as determined by HPLC 99

Table 4.3. Dietary treatments for the indicator amino acid oxidation experiment (Experiment 2 and 3) designed to determine the availability of lysine in a sample of peas 100

Table 4.4. Dietary treatments for the indicator amino acid oxidation experiment (Experiment 2) designed to determine the availability of lysine in a sample of peas .. 101

Table 4.5. Dietary treatments for the indicator amino acid oxidation experiment (Experiment 2) designed to determine the availability of lysine in a sample of peas .. 102

Table 4.6. Total BW gain, feed conversion (g feed/g gain) and lysine accretion for birds from 20 to 30 days of age fed diets for the slope ratio assay for lysine (Experiment 1)..... 103

Table 4.7. Linear regression data and availability values for live weight gain, feed conversion, and lysine accretion of 20 to 30 day old broilers fed diets for a slope ratio assay for lysine (Experiment 1)..... 104

Table 4.8. Percent ^{14}C-phenylalanine dose oxidized values for broilers fed availability diets including a basal diet (Diet 1), and incremental increases in lysine by adding free lysine (Diets 2-4), raw peas (Diet 5), cooked peas (Diet 6), and cooked peas plus lysine (Diet 7) as described in Table 2 (Experiment 2)..... 105

Table 4.9. Linear regression equations, R^2, P values and availability values determined using 19 to 32 day old male broilers using the indicator amino acid oxidation procedure (Experiment 2)..... 106

Table 4.10. Linear regression equations, R^2, P values and availability values determined using 19 to 32 day old male and female broilers using the indicator amino acid oxidation procedure (Experiment 3) 107

Table 4.11. Availability values, SEM and 95% confidence intervals for male, female and all birds as determined using the IAAO technique (Experiment 3)..... 108

LIST OF FIGURES

Figure 1.1. The theoretical relationship between oxidation of the test amino acid (lysine) and the oxidation of all other essential amino acids (e.g. phenylalanine). As dietary lysine increases towards the requirement, lysine oxidation remains low and constant, while phenylalanine oxidation decreases due to increasing incorporation into protein. Once dietary lysine is in excess, lysine oxidation increases, while phenylalanine oxidation remains constant, due to a steady rate protein synthesis. The requirement depicted by this graph is about 0.9% dietary lysine. 35

Figure 2.1. Bicarbonate retention, as a function of BW, for 7 to 42 day old broilers fed a commercial type diet free choice. The function $y = -5.5391*10^{-12}X^4 + 5.318986*10^{-8}X^3 - 1.5899*10^{-4}X^2 + 0.1587X + 13.3228$ describes the relationship between bicarbonate retention (y) and BW (x) with an $R^2 = 0.8882$. Birds were orally dosed with a prime of 1.2 μCi of $Na^{14}CO_2$ and 1 μCi of $Na^{14}CO_2$ per hour (in half hourly doses) for four hours and plateau retention values were calculated. 56

Figure 3.1: Adaptation in % ^{14}C-Phe dose oxidized from a diet containing 13 g lys/kg diet (d 0) to a diet containing 5 g lys/kg diet (d 1-7). Each data point represents the mean oxidation rate of ^{14}C-phenylalanine averaged over 4 male broilers..... 79

Figure 3.2: Adaptation in % ^{14}C-Phe dose from a diet containing 5 g lys/kg diet (d 0) to a diet containing 13 g lys/kg diet (d 1-7). Each data point represents the mean oxidation rate of ^{14}C-phenylalanine averaged over 4 male broilers. 80

Figure 4.1. Lysine availability in raw peas as determined by a slope ratio experiment using feed conversion as the response criteria in male broilers between 20 and 30 days of age (Experiment 1). Seven diets were fed, a basal diet containing 6.5 g lysine per kg diet, three diets with incremental increases in lysine as lysine-HCl, and three diets with incremental increases in lysine in the form of peas. Response criteria were plotted versus the lysine increment and the slopes of the response curves were compared to

determine the availability of lysine in peas. The availability of lysine was (-0.215/-0.306)*100 = 70.3%..... 109

Figure 4.2. Availability of lysine in raw peas as determined by a slope ratio experiment using feed conversion as the response criteria in female broilers between 20 and 30 days of age (Experiment 1). Seven diets were fed, a basal diet containing 6.5 g lysine per kg diet, three diets with incremental increases in lysine as lysine-HCl, and three diets with incremental increases in lysine in the form of peas. Response criteria were plotted versus the lysine increment and the slopes of the response curves were compared to determine the availability of lysine in peas. The availability of lysine was (-0.167/-0.272)*100 = 61.4%..... 110

Figure 4.3. Availability of lysine in raw peas as determined using a slope ratio experiment with lysine accretion as the response criteria in male broilers between 20 and 30 days of age (Experiment 1). Seven diets were fed, a basal diet containing 6.5 g lysine per kg diet, three diets with incremental increases in lysine as lysine-HCl, and three diets with incremental increases in lysine in the form of peas. Response criteria were plotted versus the lysine increment and the slopes of the response curves were compared to determine the availability of lysine in peas. The availability of lysine in raw peas was (0.291/0.415)*100 = 70.1%..... 111

Figure 4.4. Availability of lysine in raw peas as determined using a slope ratio experiment with lysine accretion as the response criteria in female broilers between 20 and 30 days of age (Experiment 1). Seven diets were fed, a basal diet containing 6.5 g lysine per kg diet, three diets with incremental increases in lysine as lysine-HCl, and three diets with incremental increases in lysine in the form of peas. Response criteria were plotted versus the lysine increment and the slopes of the response curves were compared to determine the availability of lysine in peas. The availability of lysine in the peas was (0.497/0.892)*100 = 55.7%..... 112

Figure 4.5: The % L-[1-^{14}C]phenylalanine dose oxidized for diets differing in lysine content and source of lysine. The response curve to increments of crystalline lysine is represented by the equation y = -0.379x + 22.39. The equations representing the response to raw peas, cooked peas and cooked peas plus supplemental lysine are y = -0.220x + 22.77, y = -0.156x + 22.77, and y = -0.301x + 22.77 respectively. The initial response curve was generated using four diets (diets 1 through 4 from Table 4.2), while the remaining three lines were generated using only the basal diet (diet 1) and either diet 5, 6, or 7 for raw peas, cooked peas, or cooked peas plus lysine respectively. It was assumed that if added increments of pure lysine resulted in a linear decrease in the oxidation of L-[1-^{14}C]phenylalanine, the response to lysine in the form of the test ingredient would also be linear. 113

CHAPTER 1: LITERATURE REVIEW

Background

Currently, the feed industry formulates poultry rations based on total amino acid content of feedstuffs. This is in part because the National Research Council (NRC, 1994) provides requirements for various types and ages of poultry in terms of total amino acid intake. Diets are commonly formulated with a safety margin in order to ensure that no amino acids are limiting for growth and feed conversion. These safety margins can be expensive, as adding additional nutrients is accompanied with a cost. Alternatively, failing to meet the amino acid requirements of the birds is also costly, as growth rate will be reduced and feed conversion will increase. The NRC (1998) has recently published requirements and estimated feeding values for amino acids in swine on an ileal digestibility basis. This approach reduces the amount of safety margin required to be reasonably assured the diet is complete because it takes into account the ability of the animal to digest and absorb amino acids present in the feedstuff. Therefore, low quality feedstuffs with poorly digested proteins are given a lower value that is more representative of the ability of the feedstuff to meet the metabolic requirements of the animal. Due in part to a lack of available data, the poultry industry has fallen behind in this regard.

Protein quality has been a subject of interest since the very early 1900's (e.g. Thomas, 1909). Since there are several different applications for data on protein quality (e.g. food product labeling, human nutrition, animal feed formulation, industrial applications), there have been many different approaches to quantifying protein quality.

Many of these approaches do not yield information suitable for feed formulation purposes. There are, however, several approaches that have merit in the area of animal nutrition. Further sections of this chapter will discuss the strengths, weaknesses, and potential applications of a select few methods for determining protein quality.

Prior to a discussion of methodology, it is important to clearly understand the difference between availability and digestibility. These two terms are often used synonymously, while their meanings are not exactly the same. Digestibility is a value that is determined by calculating the proportion of each amino acid that is absorbed from the feed by the animal. Availability, however, is the proportion of an amino acid in the feed that is absorbed in a form suitable to be used for protein synthesis. Various methods for the determination of digestibility and availability will be discussed in detail in the Amino Acid Availability section. The difference between these two values is the proportion of an amino acid that is absorbed in a form that the animal cannot utilize. This proportion may be large, depending on the composition and processing of the particular feedstuff. An example of amino acids that are digestible, but not available, are the products of the Maillard reaction. This reaction occurs when a feed or food product is overheated, causing the amino group of amino acids to bind a carboxyl group of a reducing sugar. This reaction occurs to the largest extent between lysine and reducing sugars. Some of the lysine that undergoes this reaction will be completely indigestible, while some will be absorbed, but will remain unused for protein synthesis.

Since many of the most common protein sources in animal feeds are heat-treated during normal processing (e.g. soybean meal, canola meal, meat meal, fish meal), it is beneficial to be able to determine the availability, rather than digestibility, of these protein meals. The slope ratio technique, described by Batterham et al. (1979; 1984;

1986) uses various growth data to determine the availability of amino acids in feedstuffs. The strengths and weaknesses of this method are described in a following section (Amino Acid Availability).

The research presented in this thesis was designed to develop a less expensive, time-consuming, and laborious method of determining amino acid availability in feedstuffs for poultry using the Indicator Amino Acid Oxidation (IAAO) technique. Prior to a discussion of the current research, it is useful to review the currently available methods for determining amino acid availability and digestibility in animals. As well, the strengths and limitations of the IAAO method will be discussed.

Amino Acid Availability

Biological Value

One of the earliest methods of measuring protein quality was developed by Thomas (1909), and revised by Mitchell (1924). The biological value (BV) method that resulted is still used for ranking samples in order of protein quality. The method is best described by the equation used to calculate BV:

Equation 1: $$BV = \frac{NI - (FN - MFN) - (UN - EUN)}{NI - (FN - MFN)}$$

(Evans and Witty, 1978)

Where NI is nitrogen intake, FN is fecal nitrogen, MFN is metabolic fecal nitrogen, UN is urinary nitrogen, and EUN is endogenous urinary nitrogen.

From this equation, one can see that BV essentially measures the amount of nitrogen retained by the body as a proportion of the nitrogen absorbed from the feed.

One limitation of this procedure is the estimation of MFN and EUN. The MFN value is typically obtained through feeding a nitrogen-free diet to a set of animals similar to the test animals and measuring the nitrogen content of the feces. While this approach helps to account for the fecal loss of endogenous nitrogen, it has been shown to be inaccurate due to effects of diet on endogenous secretions. This is because dietary protein, energy, and fibre have all been shown to affect endogenous nitrogen secretion (Partridge et al., 1982; Zebrowska, 1985), and a protein free diet cannot match the test diet for these nutrients. These endogenous secretions include sloughed intestinal cells and enzymatic secretions, which increase in response to the presence of certain nutrients. Endogenous urinary nitrogen has similar drawbacks. Endogenous urinary nitrogen is typically measured through feeding of a nitrogen-free diet as well, which has a dramatic impact on amino acid metabolism, and hence urinary nitrogen excretion. The decrease in protein turnover, as well as adaptive enzyme changes, will reduce catabolism of amino acids in an effort to conserve them for protein synthesis or production of other metabolic products (e.g. serotonin, catecholamines), which will provide less nitrogen for urea or uric acid synthesis (Groff et al., 1985). As urea or uric acid compose the largest proportion of the nitrogen excreted by the animal, reduction of this metabolic product will dramatically impact the values for EUN and hence the BV of the feedstuff. These adaptive changes have been shown to occur to some extent by 4 hours after the test diet is changed. Since these adaptations result in a metabolic state that is not representative of the obligatory nitrogen loss that would occur with a diet that has amino acids, EUN is not an accurate correction factor.

The value that results from the determination of BV is only representative of the availability of the first limiting amino acid. Although this number is of value, and the availability of the next limiting amino acid can be determined through supplementing the

diet with the first limiting amino acid, this method is limited by the fact that the researcher does not know which amino acid is first limiting. The main advantage, which made BV a valuable tool in the early 1900's, is that the determination of individual amino acid concentrations is not required. Nitrogen values for feed, feces, and urine are all that are required in determining BV. While this simplifies the method, it also contributes to error, since not all nitrogen in a feed sample is from protein. Other sources of nitrogen include nucleic acids, nitrates, and other naturally occurring compounds. Knowing this, it is also important to point out that changes in amino acid profile due to microbial action become irrelevant, and allow fecal collection rather that collection of ileal digesta.

Net Protein Utilization

Several methods were developed as improvements on the BV method. Of these, net protein utilization (NPU), developed by Bender and Miller (1953) is the most relevant. This method essentially removes the second term in the denominator of Equation 1. The new equation represents the retained nitrogen as a proportion of the total nitrogen intake, rather than a proportion of absorbed nitrogen. This helps to compensate for completely indigestible protein, which is not accounted for with BV values. For example, a highly available protein source with 20% protein would have the same BV as a similar protein source with 30% protein if the additional 10% protein were indigestible. The NPU, however, would be lower for the second protein source. Essentially, NPU is a more useful value for feed formulation purposes than BV, since the total protein in a feedstuff is often known, when the digestible protein in the sample is not known. However, NPU has the same drawbacks as BV regarding MFN and EUN.

Protein Efficiency Ratio

Osborne (1919) described another method to determine the quality of protein sources. This method was termed protein efficiency ratio (PER). PER was determined using the following equation:

Equation 2: PER = Weight gain / Protein intake

(Evans and Witty, 1978)

One can see from the equation that PER closely resembles the calculation of feed efficiency commonly used in the poultry industry to determine the overall quality of complete feeds. Essentially, animals are fed the feedstuff, supplemented with essential non-protein nutrients, for a period of time, and the ability of the protein source to promote weight gain is determined. Diets must be isoenergetic and isonitrogenous in order to obtain comparable results. As well, the animals used must be genetically similar, and at the same stage of growth in order to ensure similar amino acid requirements. Chapman et al. (1959) and Derse (1957) developed standardized procedures for determining PER for Canada and the USA respectively.

While PER has been used as a standard measure for determining the quality of proteins for food labeling purposes for many years, there are many criticisms of this method. The first is that animals fed different protein levels will yield different PER values (Hurt et al., 1974). For example, a rat fed a 10% protein diet will have a lower PER than a rat fed 15% of the same protein. This is thought to be due to the changing proportion of the protein intake that is partitioned to maintenance. At 10% protein, a large proportion of the protein intake is diverted to maintaining tissues (ie not promoting growth) while at 15% protein, proportionately more protein is available for growth as and less is used for maintenance. As well, weight gain that is not attributed to protein (e.g. fat

deposition) is not accounted for in the above equation. These factors are accounted for in the standardized procedures described by Chapman et al. (1959) and Derse (1957). PER also only accounts for the first limiting amino acid, as protein accretion will be determined by that amino acid. The advantage of this method is that nitrogen determination is only required on feed samples, eliminating much of the lab work involved in determination of BV.

Net Protein Ratio

A further improvement to PER is the net protein ratio (NPR) described by Bender and Doell (1957a; 1957b). This method employs a control group, which is fed a protein-free diet, and corrects the weight gain in the above equation by adding the weight loss of the control group to the gain of the test group. This is an attempt to remove the effect of protein level by correcting for maintenance requirements (Evans and Witty, 1978). However, protein deposition has been shown to be curvilinear in response to intake (McLaughlan and Keith, 1974) resulting in an effect of protein intake on NPU values as well. Neither PER or NPU result in a number that can be used for feed formulation, but can be useful in ranking samples in order of availability of the first limiting amino acid. These methods are more suitable for use in human nutrition and regulation of food products that for use in the animal feed industry.

Nitrogen Balance

Nitrogen balance experiments have traditionally been used to determine both the requirement of an animal for an amino acid and the availability of that amino acid in the feedstuff. In a nitrogen balance experiment, a test diet is fed to the subject for an adaptation period, typically 9-12 days (Rand et al., 1981). This allows for adaptation of

both the catabolic enzymes and the urea pools in the body (Rand et al., 1981). Nitrogen balance is then determined daily over a period of several days, in order to reduce the variation found with a subject on different days (Rand et al., 1981). Balance is determined by calculating the nitrogen intake of the individual and subtracting the total excretion of nitrogen from all sources (i.e. urine and feces). The nitrogen balance over several protein intakes both above and below maintenance is graphed. The nitrogen intake at which nitrogen balance is zero is considered the subject's requirement for nitrogen (Rand et al., 1981). As the slope of the graphed line is assumed to be linear below the requirement, the slope of this line is considered the efficiency of utilization of the protein (Rand et al., 1981).

One criticism of nitrogen balance studies is that due to adaptive changes in catabolic enzyme activity, the relationship between nitrogen intake and nitrogen excretion is not linear below the requirement (Young et al., 1973). This would result in inaccurate results concerning the efficiency of nitrogen utilization. As well, the length of time required to perform an experiment with several data points both above and below the requirement would exclude some subjects from this type of experiment. Broiler chicks would be among the excluded subjects, as it is impossible to maintain a steady physiological state in rapidly growing birds.

Protein Digestibility-Corrected Amino Acid Score

Of the large number of chemical and enzymatic methods that have been developed for the determination of protein quality, a recent adaptation of the amino acid score shows the most promise. The amino acid score is determined by analyzing the amino acid content of the food or feedstuff and comparing the results to the requirement

for the human or animal for which it is intended. The protein digestibility-corrected amino acid score (PDCAAS) was accepted by the FAO/WHO in 1989 as the standard by which protein quality should be expressed (Schaafsma, 2000). This method involves first determining the amino acid score. The PDCAAS method then adds a term for the true digestibility of the amino acid, as determined in rat fecal digestibility trials. This results in a series of numbers that represent the ability of the digestible amino acid content to meet the requirement of those who will consume the product. The lowest value obtained is selected, and that number is considered the PDCAAS.

While there are some criticisms of the PDCAAS method, it is an improvement over previous methods. The first criticism is that the requirement chosen for use in the standardized method of the WHO (Schaafsma, 2000) are those determined for pre-school aged children that were recovering from a state of malnutrition. These requirements cannot be taken as representative of all human populations. As well, rat trials that are used to determine availability may or may not be representative of an average human's ability to digest the protein source. Finally, digestibility values are not as accurate and availability values for reasons described in the previous sections. The merits of fecal digestibility, as compared with ileal digestibility are discussed below. With refinement, the PDCAAS method shows promise for use in human nutrition and regulation of food products and labeling, but is of little use to the animal feed industry, as a single value doesn't allow for prediction of the additive value of feedstuffs. If PDCAAS values for all amino acids in a feedstuff were maintained, the resulting values would not be an improvement on the ileal digestibility values used in formulating swine rations. It would, however, improve current formulation methods in poultry. In animal nutrition, the PDCAAS is essentially equivalent to ileal digestibility, provided all of the amino acids are maintained.

Digestibility

The most common method for determining protein quality for the animal feed industry is the use of digestibility values. Digestibility can be determined in a number of ways. These can be divided into fecal vs. ileal digestibility and true vs. apparent availability.

Fecal digestibility has one advantage over ileal digestibility; the collection of fecal matter is much simpler and less invasive than the collection of ileal digesta (Sarwar et al., 1989). The collection of ileal digesta requires either the death and dissection of the animal, or the surgical insertion of an ileal cannula and the subsequent recovery time (Sarwar et al., 1989). The advantage of ileal digestibility is that the values obtained are much more representative of the actual value of the protein to the animal. The microbial population, located primarily in the large intestine and ceca, has been shown to alter the amino acid profile of the digesta as it passes though. Therefore, fecal digestibility is not representative of the actual amount of each amino acid that is not absorbed by the animal (Sauer et al., 1989). Since the absorption of amino acids in the large intestine is thought to be either minimal or zero, the extraction of digesta from the ileum allows determination of the amount of unabsorbed amino acids prior to the action of the colonic microbial population. Poultry species are capable of retrograde peristalsis (Whittow, 2000) , which could result in digesta from the ceca moving back into the ileum, so this method has not been accepted for use in poultry.

An adaptation of the ileal digestibility technique in poultry is the use of cecectomized Leghorn roosters (Whitacre and Tanner, 1989). These roosters have

undergone surgery to remove the ceca, which is the primary site of microbial action in the chicken. Some experiments have shown that ileal digestibility and fecal digestibility in cecectomized birds are similar (Green at al., 1987), while other have shown differences for some amino acids (Han et al., 1990). In general, this technique has been accepted for the determination of amino acid digestibility in poultry.

Sibbald and Wolynetz (1986) developed a method for determination of metabolic fecal nitrogen, which did not entirely rely on protein free diets. This method was used with the true metabolizable energy (TME) method for determining energy and amino acid digestibility in feedstuffs. Briefly, diets were precision-fed, and excreta was collected to determine the disappearance of energy and amino acids during digestion. Different diets with graded levels of the test feedstuff were fed to the birds. Through multiple linear regression, the level of nitrogen excretion at zero feed intake, and at zero feed intake corrected to zero nitrogen balance, was determined and used to compensate for metabolic fecal nitrogen and endogenous urinary nitrogen. This method is an improvement over the feeding of protein-free diets, but is also imperfect for adjusting digestibility values. This is because the endogenous secretions and rate of cell soughing may not be the same at zero feed intake as it was at the test level of feed intake.

The determination of apparent digestibility is similarly much more simple than the determination of true digestibility. Both of these values can be obtained for either fecal or ileal digestibility. Apparent digestibility involves determining the amount of each amino acid that is excreted by the animal. True digestibility is more complicated in that it requires adjustment of fecal or ileal digesta amino acid levels for endogenous losses of each amino acid. This is more complicated due to methodological problems involved in the estimation of endogenous losses. Several methods have been employed

to attempt to estimate losses of this sort. The first is the feeding of a protein-free diet to a similar animal, and subsequent determination of the amount of each amino acid in the feces or ileal digesta. This method is not completely accurate, in that the level of protein, fiber and energy in the diet has been shown to increase the amount of endogenous secretions in the intestinal tract, as well as the rate of intestinal cell sloughing (Partridge et al., 1982).

The most accepted method at present for determining the digestibility of amino acids is, therefore, true ileal digestibility. The advantages of this method are that the digestibility of all amino acid are determined at once, and that the values obtained allow of accurate prediction of the value of combined feed ingredients. As well, the values obtained are not in reference to the requirements of any one species at a given stage of development, and are therefore applicable to all similar animals assuming that their ability to extract amino acids from the feed is not dramatically different (e.g. hatchlings vs. adult roosters). This assumption may not be applicable in all circumstances, however, as values obtained by caecectomized Leghorn roosters are applied to diets fed to day old broiler chicks. The drawback of digestibility values, as described earlier, is that they do not account for the ability of the animal to use the amino acid for protein synthesis once it has been absorbed.

Slope Ratio Technique

The slope ratio method, developed primarily by Batterham's group in Australia (Batterham et al., 1979; 1984; 1986; van Barneveld et al., 1994a; 1994b; 1994c) uses several different indices to determine the growth response to graded levels of a test amino acid. This technique has been successfully applied to determine the metabolic

availability of essential amino acids in swine, poultry and rats. Test diets are formulated to be isoenergetic and isonitrogenous, and complete except for the test amino acid. The amino acid of interest is added in graded levels in a purified form to some diets and as the test ingredient in other diets. Typically 6 to 8 diets are formulated containing either the purified amino acid (assumed to be 100% available) or the test ingredient. Responses are determined using feed conversion, weight gain and protein or amino acid accretion. These indices have been shown to have a linear response to increases in the level of a test amino acid provided the test amino acid is below the requirement. A linear regression is performed to determine the equation for the response line for both the purified amino acid and the test ingredient diets. The slope of the test ingredient line divided by the slope of the purified amino acid line, expressed as a percentage, is the availability of the test amino acid in the feedstuff.

This method is currently the most accurate for measuring the availability of amino acids in feedstuffs, and results in values that can be used to predict the additive feeding value of ingredients. The drawbacks of this method are mainly the cost and time required to perform the analysis. Each set of six to eight diets (Batterham et al., 1979), and the accompanying carcass analysis yields an availability value for one amino acid in a given feedstuff. The time required to perform the experiment is typically 10 days (Batterham et al., 1979; 1984; 1986), but analysis of the samples taken can take much longer and may be very expensive. Typically, with eight diets and a minimum of four to six animals per treatment, these experiments would require 32 to 48 animals per amino acid availability value. While this method is accurate and results in usable numbers, a less expensive and more rapid alternative would be desirable.

Direct Oxidation

Prior to the development of the IAAO approach, the direct oxidation method was developed. Because the IAAO method was developed to avoid problems associated with this approach, it is worth briefly discussing the method.

Brookes et al. first conceived oxidation (1972), and used to quantify the oxidation of lysine in rats. The method involved cardiac injection of a radio-labeled solution of the amino acid under study in a bolus dose, and collection of the radioactivity that was exhaled by the animal. The theory involved was that the oxidation of the labeled amino acid would be indicative of the fate of the naturally occurring amino acid. Therefore, if lysine was deficient in the diet, both the naturally occurring lysine and the labeled lysine would be oxidized at a low rate. If lysine were sufficient, the collection of the label would be higher, due to greater oxidation of all sources of lysine. More recently, Zello et al. (1990) successfully used the direct oxidation approach to quantify the requirement for phenylalanine in humans. A primed constant infusion of L-[1-^{13}C]phenylalanine was used, and diets both deficient and excess in phenylalanine content were fed.

A limitation of the direct oxidation method is that it is not applicable to all indispensable amino acids (Brunton et al., 1998). This is due to the condition that the amino acid under study must not be part of any significant pathway other that oxidation and protein synthesis. An example of an inappropriate amino acid is methionine that has a complex metabolic pathway that would make oxidation

results difficult to interpret. A second limitation is that in requirement studies, deficient diets must be fed (Brunton et al., 1998). This becomes problematic in human studies where the use of ^{14}C is unethical. The isotope commonly used in human studies, ^{13}C, is stable and therefore not dangerous to the test subjects, but also naturally occurs in the environment. Because of this, large amounts of the tracer must be used in order to overcome background levels, and therefore very low dietary intakes cannot be studies.

To overcome the limitations of the direct oxidation method, the IAAO method was developed. Since the indicator amino acid is that which is measured, the test amino acid can have several metabolic fates without affecting the results of the experiment. As well, the level of the indicator amino acid can be maintained at relatively high levels, enough to overcome background levels of ^{13}C, without affecting the concentration of the test amino acid that is fed. It is possible, therefore, to study amino acid metabolism at zero intake of the test amino acid. The IAAO method is described in more depth in the next section.

Indicator Amino Acid Oxidation

Prior to discussion of the application of the indicator amino acid oxidation (IAAO) method to the determination of amino acid availability, one must consider the IAAO method itself and the pros and cons associated with it.

IAAO Procedures

The indicator amino acid oxidation (IAAO) technique has been developed for use in humans (Zello et al., 1990; 1993; 1995), pigs (Ball and Bayley, 1984; 1985; House et al., 1997; 1998), and chickens (Tabiri et al., 2002a; 2002b). Currently this method is used to determine the requirements of humans and animals for essential amino acids or total protein. The theory on which this method relies on is that the utilization of the indicator amino acid is proportional to the change in protein synthesis that is caused by the test amino acid.

Protocols in chickens, pigs and humans involve feeding diets formulated to be isoenergetic, isonitrogenous, and complete except for the test amino acid. The test amino acid is fed in graded levels, which are spaced to widely bracket the expected requirement. An example of the relationship between the test amino acid (lysine) and the indicator amino acid (phenylalanine) is depicted in Figure 1.1. As the level of the test amino acid increases towards the requirement for protein synthesis, the rate of oxidation of the test amino acid remains low and constant while oxidation of the indicator amino acid decreases. This is due to a reduction in the rate of catabolism of the test amino acid designed to conserve it when it is deficient, and incremental increases in protein synthesis that occur with increases in the test amino acid. Once the requirement has been surpassed, the oxidation rate of the test amino acid increases with each incremental increase in dietary level of that amino acid. Since increases in the test amino acid no longer support an increase in protein synthesis, oxidation of the indicator amino acid becomes constant past the requirement for the test amino acid. The oxidation rates determined using the IAAO method are plotted, and through regression analysis the inflection point is determined. The inflection point of the graph, the point at which an

increase in the test amino acid no longer results in a decrease in the oxidation of the indicator, represents the requirement of the test amino acid for protein synthesis.

The IAAO method was also first conceived by Brookes et al. (1972). Brooks et al. used a bolus-dose cardiac-injection method to test diets differing in lysine content. As well as using the direct oxidation method, Brookes et al. used L-^{14}C-methyl-methionine to quantify lysine oxidation. L-^{14}C-methyl-methionine was shown to be inappropriate for IAAO studies, due to the large contribution of methyl groups from methionine to other molecules. The experiment was performed as a single bolus dose injection into the heart of the rats. Later experiments on substrate oxidation showed that bolus dose oxidation studies were inappropriate for determining the rate of oxidation of an amino acid (Wolfe, 1992) or any other substrate (Issekutz et al., 1968). Research from the University of Guelph were designed to adapt the IAAO method for measuring oxidation rates in pigs (Kim et al., 1983a; 1983b; Kim and Bayley, 1983; Ball and Bayley, 1984; 1985; 1986; Ball et al., 1986; Lin et al., 1986a; 1986b). The method used by these authors involved two bolus oral doses of the indicator during feeding of diets varying in the level of various test amino acid.

Currently, there are two method used to calculate the rate of oxidation of the indicator amino acid. The first method has been used primarily animal studies and radioactive isotopes (Tabiri et al., 2002b; Coleman et al., 2003). This method uses the collection of labeled CO_2 (DPM), as a proportion of the amount of the indicator amino acid infused (DPM). Collection of the label is performed by collection of the CO_2 in the breath. This method allows calculation of the percentage of the infused dose that is oxidized by the animal. The second method requires determination of the rate of flux. This method is more commonly used in humans (Zello et al., 1990; 1995) and in neonatal

piglet models (Bertolo et al., 1998). This method uses breath collection to determine the percentage of phenylalanine that is oxidized, and the flux rate to calculate the daily oxidation of phenylalanine in mg per kg BW per day. Flux is measured by sampling the plasma pool and performing kinetic calculations. The later approach requires sampling of the plasma pool in order to calculate the pool size and flux rate from the infusion of the isotope. The former approach is less difficult, and results in values that can be used to calculate requirements, but provides no kinetic data for the indicator amino acid.

Criticisms of the IAAO Procedure

There are several assumptions made in the IAAO method that should be explained prior to further discussion of its merits. The first, and most important, is the use of single body amino acid pool kinetics for each amino acid. Calculations of kinetics made by most researchers (Zello et al., 1990; 1993; 1995; Ball and Bayley, 1985; 1986; House et al., 1998; Bertolo et al., 1998), regarding flux and pool size are made assuming that there is a single pool for both the test and indicator amino acids. The assumption is, essentially, that infusion of the isotopically labeled amino acid, and sampling of that amino acid, occurs in a single, instantaneously mixing, and homogenous pool. This is clearly not the case for amino acids, as models that employ a multiple pool model have shown different rates of appearance and disappearance from various pools, and that the oxidation of the indicator amino acid occurs primarily in the liver, suggesting that sampling of labeled CO_2 would be higher in the liver than in the pool that is sampled (plasma). As well, instantaneous mixing of an infused substrate is unlikely, although for the purposes of steady state oxidation experiments, this assumption likely does not affect the resulting values dramatically (Wolfe, 1992).

Another assumption made is that the indicator amino acid used in the experiment has only two significant metabolic fates; either retained for protein synthesis or irreversibly oxidized to labeled CO_2. There can be no other significant metabolic products produced, or the interpretation of oxidation data would be difficult. Two amino acids have been shown to be appropriate, lysine and phenylalanine. Lysine has no metabolic fates other than oxidation and protein synthesis, but has a large and variable pool size that may yield high variation in oxidation results. Phenylalanine is the most commonly used indicator amino acid, because it has a small, tightly controlled body pool. Although oxidation of phenylalanine occurs through tyrosine, it has been shown that less than 3% of the total radioactivity infused as ^{14}C-phenylalanine ends up in tyrosine when dietary tyrosine is in excess (Zello et al., 1990). The carboxyl carbon of phenylalanine can also be metabolized into melanin and thyroid hormone, but these pathways are thought be negligible in oxidation studies (Zello et al., 1990). Branched chain amino acids, as well, fit the above criteria for an appropriate indicator amino acid (Kurpad et al., 2003a; 2003b), however they have been shown to influence protein synthesis. Leucine, isoleucine and valine, however, are oxidized to a significant degree outside the liver (i.e. muscle tissue), and thus their oxidation may not provide an appropriate index of the amino acid profile that reaches the liver.

The site of labeling of the indicator also has implications in the interpretation of the results of an oxidation study. The indicator must be labeled on a carbon that is oxidized only to CO_2. For this reason, the most commonly used isotope L-[1-^{14}C]phenylalanine. The carboxyl carbon of phenylalanine is irreversibly oxidized to $^{14}CO_2$ in all cases, save when tyrosine is produced. Other isotopes, such as L-[U-^{14}C]phenylalanine, L-[^{15}N]-phenylalanine, and various ^{3}H isotopes may be used to trace phenylalanine metabolism, but are not appropriate for oxidation studies because the label

may be incorporated into several metabolic products depending on metabolic state of the animal.

Millward and Rivers (1988) discussed the merits of the direct oxidation method of determining amino acid requirements, pointing out methodological limitations. Several of these sources of error are avoided through the use of the indicator method, while some still apply. Their first criticism of isotopic techniques in general was that retention of the labeled bicarbonate produced, in various body pools and metabolic products, varies with metabolic state. For example, exercise has been shown to increase irreversible loss of infused bicarbonate in humans (Barstow et al., 1990). Any error in correction for label recovery would serve to skew rates of oxidation in any substrate oxidation experiment. To circumvent this problem, studies to determine the bicarbonate retention factors for humans (Zello et al., 1993), swine (House et al., 1998) and poultry (Tabiri et al., 2002a) have been performed under the specific environmental conditions used during the experiment. Controlling for factors that alter metabolic rate would reduce the difference in bicarbonate retention and improve the accuracy on the substrate oxidation study. Some researchers believe that bicarbonate retention must be measured in individual subjects on the day of the oxidation experiment in order to achieve accuracy (Hoerr et al., 1989), while others believe that characterization of a population using the same environmental and dietary condition is sufficiently precise (Irving et al., 1983). This topic will be discussed further in the following section.

A further criticism of tracer studies involves problems with the labeled CO_2 collection procedure developed and used by some researchers. Procedures involving "Douglas bag" collection of expired air were specifically mentioned by Millward and Rivers (1988). Douglas bags were used in several experiments in order to collect air from

the subjects. These bags were found to leak, and the short period of collection may not be representative of the entire experimental period. This problem has been avoided in animal experiments, where placing the animal in a polycarbonate chamber and forcing air through a series of bottles that contain monoethanolamine to achieve total collection. Monoethanolamine binds the CO_2 that passes through, and can be used to determine the total radioactivity exhaled.

The final criticism that applied to the indicator method is that the experimental period used was too short to accurately represent the daily variation in oxidation rate of amino acids. This is thought to be a problem, particularly because the experiments are typically performed in the fed state and meals are often given that do not necessarily represent the "normal" intake of nutrients. An example of this is shown in Young et al. (1987) where half the daily intake of the subjects was fed over a 4 h period. This criticism is more pertinent to isotope balance studies, but also applies to the IAAO method. Studies in animals can avoid this source of error by constant infusion of a nutrient solution, as employed by House et al. (1997; 1998) and Bertolo et al. (1998) in neonatal piglets.

Several of the criticisms of the direct oxidation technique are avoided by use of the IAAO method. Most notably, the dietary level of the test amino acid can be reduced much further with IAAO as compared to the direct oxidation method. The rather large dose of labeled amino acid that is required, particularly with the use of stable isotopes, can result in the majority of the dietary test amino acid intake being supplied as label. This no longer constitutes a tracer amount of the amino acid. With the IAAO method, the test amino acid can be reduced to zero without affecting the level of the tracer used.

In order to properly use the IAAO method, several considerations must be made in the experimental protocol. The diets must be formulated to be isonitrogenous, isoenergetic, and contain a constant and excess amount of the indicator amino acid. In the case of phenylalanine, both phenylalanine and tyrosine must be in excess in all diets. Deficient tyrosine will result in conversion of labeled phenylalanine to tyrosine and consequently affect the oxidation results. Zello et al. (1990) showed that with excess tyrosine, phenylalanine conversion to tyrosine was less than 3% and is not affected by amino acid intake. Dietary phenylalanine levels must be held constant in order to achieve a constant dilution of the isotope in body pools. Experimental protocols in humans (Zello et al, 1990; 1993; 1995) and neonatal pigs (House et al., 1997; 1998; Bertolo et al., 1998) have achieved this constant dilution of phenylalanine by reducing the amount of phenylalanine in the diet fed on the experimental day by the amount of tracer infused. Experiments in older pigs (Ball and Bayley, 1985; 1986) balanced the diets for phenylalanine and tyrosine, but did not account for the labeled dose of phenylalanine the piglets received on the day of the experiment. If the dose of phenylalanine is truly a tracer amount, then leaving it unaccounted for would have no significant impact on its dilution.

IAAO Availability Method

The direct oxidation method, as described earlier, was used in a previous attempt to develop a rapid method for determining lysine availability in feedstuffs (Ball et al., 1995). In this experiment, pigs were fed diets with excess lysine, and the labelled amino acid (L-[U-^{14}C]lysine and DL-[1-^{14}C]lysine) was expected to increase with increasing lysine availability. The rates of oxidation of the labelled amino acids in that trial were higher for the feedstuffs than the control (purified lysine) diet, indicating that the

availability of lysine in those feedstuffs was greater than 100%. The method was not considered valid, and further work was suggested.

With the above discussion in mind, the IAAO method has the potential to be applied for the determination of metabolic availability of amino acids in poultry. The theory is much the same as that of the slope-ratio method. Since the oxidation of the indicator amino acid decreases linearly with addition of the test amino acid at levels below the animal's requirement, a slope-ratio assay that is based upon the rate of oxidation of the indicator is plausible. The oxidation of the indicator amino acid can be used as a response criteria in a slope ratio assay in order to reduce the time required for the trial itself and sample analysis.

Research Objectives

The objective of this research was to develop the IAAO method in broilers, and to test the validity of this method for the determination of the metabolic availability of indispensable amino acids in poultry feedstuffs.

To develop the IAAO method in broilers, the bicarbonate retention factor was required for various ages under investigation. The bicarbonate retention factor was determined, through a primed, constant oral dose protocol, for birds at 0, 7, 14, 21, 28, 35, and 42 days of age. As bone development is thought to affect bicarbonate retention, the cross-sectional area and densities for cortical, trabecular and total bone at the midpoint of the femur was determined through Quantitative Computed Tomography analysis. These data were analyzed to determine if there was a relationship between bone

development and bicarbonate retention. A regression equation to predict bicarbonate retention using body weight was also developed for use in future studies.

The second experiment was designed to determine the priming and hourly oral dose levels of L-[1-14C]phenylalanine that resulted in the most rapid attainment of plateau during an oxidation study. Oxidations were performed using a commercial-type diet, three different priming and three different hourly oral doses. The time to plateau was determined in to select the appropriate priming to oral dose ratio for broiler chickens.

The third experiment was designed to determine the adaptation period required for broilers to metabolically adapt to varying levels of the test amino acid in the diet. Birds were first adapted to a diet either sufficient or deficient in lysine for 7 days. An oxidation was performed, and the diet was changed to the other diet. The time required to reach a new and stable rate of oxidation after the change of diet, and the intra-individual variation was determined for changes in diet both from sufficient to deficient and from deficient to sufficient.

The final experiment was designed to validate the IAAO method for determining amino acid availability. A slope-ratio trial was performed to determine the availability of lysine in peas for both male and female chicks between 20 and 30 days of age. The IAAO method was then used to determine the availability of lysine for male birds between the ages of 19 and 32 days. The results of the two experiments were compared to determine if the IAAO method could be used to determine the availability of amino acids in feedstuffs.

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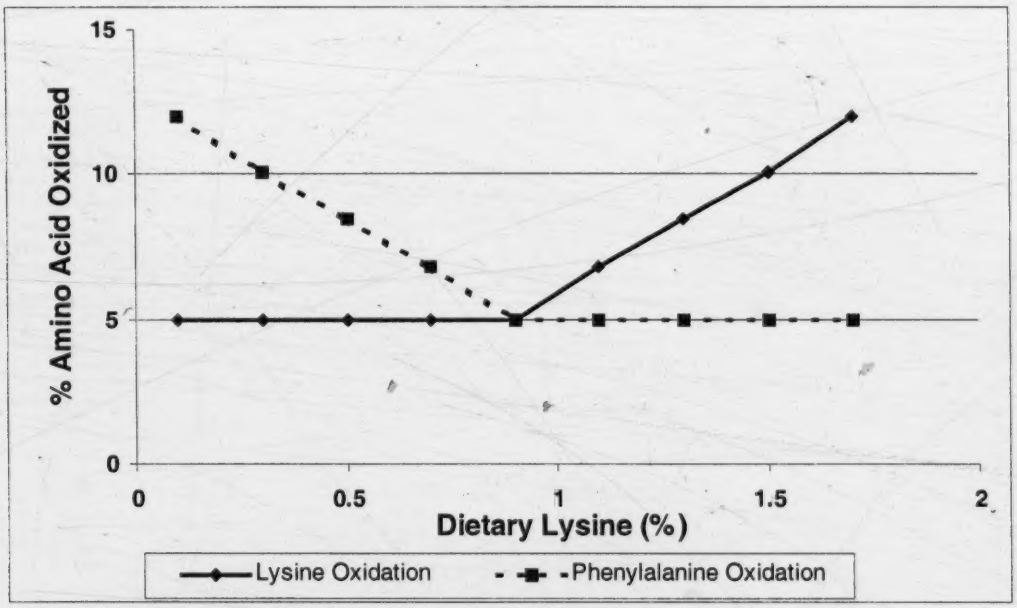

Figure 1.1. The theoretical relationship between oxidation of the test amino acid (lysine) and the oxidation of all other essential amino acids (e.g. phenylalanine). As dietary lysine increases towards the requirement, lysine oxidation remains low and constant, while phenylalanine oxidation decreases due to increasing incorporation into protein. Once dietary lysine is in excess, lysine oxidation increases, while phenylalanine oxidation remains constant, due to a steady rate protein synthesis. The requirement depicted by this graph is about 0.9% dietary lysine.

CHAPTER 2: BICARBONATE RETENTION AND BONE DENSITY

Introduction

Isotopically-labeled substrates are often used to trace metabolic pathways in order to determine the fate of compounds fed or infused into animals and humans. In many studies, the substrate is labeled within the infused molecule on one carbon that has a single metabolic fate, which is often the production of labeled carbon dioxide through oxidation of the substrate. The $^{14}CO_2$ produced is then measured in the animals' breath and used to quantify the rate of oxidation of the substrate. The measurement of breath $^{14}CO_2$ is not a completely accurate method of estimating oxidation rate. Many studies have shown that the recovery of $^{14}CO_2$ infused as $NaH^{14}CO_3$ is not complete during a practical timeframe due to exchange between bicarbonate pools with varying turnover rates and incorporation into other metabolic products (e.g. urea/uric acid, amino acids, lipids, glycogen, hydroxyapatite, etc.) (Groff et al., 1985). To accurately determine the rate of oxidation of these substrates, it is crucial to develop reliable information on the retention of labeled C in the animals' bicarbonate pools under the specific conditions applicable to the experimental procedure.

Tabiri et al. (2002a) investigated the bicarbonate retention of broiler breeder pullets prior to the onset of sexual maturity. Other studies have shown that factors such as age and metabolic state have an impact on retention (Armon et al., 1990; Barstow et al., 1990). Therefore, the results found by Tabiri et al (2002a) may not be applicable to growing broilers. As well, broilers at different stages of development are likely to have

different retention factors due to changing metabolic rate associated with changes in growth rate and breast muscle deposition (Barstow et al., 1990).

The site of infusion of labeled bicarbonate has been the subject of several studies (Downey at al., 1986; Hoerr et al., 1989; Benevenga et al., 1992). Venous infusion, the most common approach, was compared to arterial infusion (Downey et al., 1986; Benevenga et al., 1992) and oral dosing of the bicarbonate tracer (Hoerr et al., 1989) with no significant differences between sites of infusion. Interestingly, Hoerr et al. (1989) showed that none of the orally dosed bicarbonate was liberated prior to absorption in the small intestine. Absorption was thought to occur via intracellular diffusion.

Poyart et al. (1975a) investigated bone as a sink for circulating bicarbonate from the blood in rats. They found that 30% of the bicarbonate in the blood that is supplied to the bone is transferred to a labile bicarbonate pool. This pool is thought to be associated with the bone water rather than the structural bicarbonate, which has a much slower turnover rate. The amount of bicarbonate entering the labile bone water pool is thought to be limited by the blood flow to the bone, rather than the capacity of the bone to sequester bicarbonate (Poyart et al., 1975b). In rapidly growing birds, bone deposition could as well add to the amount of bicarbonate that is fixed through hydroxyapatite deposition (Whittow. 2000).

The present experiment was performed in to develop a correction factor that could be applied to future, steady state oxidation experiments on broilers between 0 and 42 day-old in a fed state. In order to identify any potential effect of the stage of bone development on bicarbonate retention, certain bone traits were determined for birds of the same age and body weight. The total, trabecular and cortical bone mineral densities, as

well as cross sectional areas were determined by quantitative computed tomography at the midpoint of the femur, and used as an indicator of bone size and mineralization (Saunders-Blades et al., 2003).

MATERIALS AND Methods

Stock and Management

All procedures were reviewed and approved by the University of Alberta Faculty Animal Policy and Welfare Committee, and were in accordance with Canadian Council on Animal Care (1993) guidelines. Two hundred male Ross 308 chicks were obtained from a commercial hatchery on the day of hatch. The chicks were transported to the Metabolic Research Unit at the University of Alberta Edmonton Research Station. The birds were housed in a single floor pen with an initial stocking density of 11.4 birds/m^2. Temperature was maintained according to an industry standard temperature schedule, and adjusted according to bird behaviour. Birds were provided feed and water ad libitum for the duration of the experiment. A commercial-type starter diet was fed from 0-21 days, and a grower from 22-42 days (Table 2.1). A lighting schedule of 23 hours light, 1 hour dark per day was employed for the duration of the experiment.

Experimental Design

Birds were individually weighed weekly to determine the average population weight. Beginning on day 0, and at weekly intervals, 16 birds were selected that were within 5% of the mean population weight. Four of the selected birds were designated for use in the bicarbonate retention experiment, while the remaining 12 were killed via cervical dislocation and set aside for bone analysis by quantitative computed tomography (QCT).

The four birds selected for the bicarbonate retention trial were placed in polycarbonate oxidation chambers and allowed free access to feed and water at all times. The oxidation chambers and breath collection apparatus used here have been previously validated and described by Tabiri et al. (2002a).

Before the experiment, each bird was given a priming dose of approximately 1.2 μCi of $NaH^{14}CO_3$ by oral gavage. After 15 minutes, the birds were given the first of eight half-hourly doses of 1 μCi of $NaH^{14}CO_3$ in 0.5 ml of saline. Due to the difficulty in accurately determining the amount of ^{14}C in a bicarbonate stock sample, and the possibility of liberation of the label as $^{14}CO_2$ over time (Wolfe, 1992), radioactivity was determined on each experiment day. The isotopically-labeled bicarbonate was mixed into a saline solution and stored at 4 degrees C in a sealed glass bottle for the duration of the experiment. Syringes were filled with the appropriate amount of isotope solution, and their weights recorded. Regardless of BW, all birds were given approximately the same amount of $NaH^{14}CO_3$ by oral gavage. Oral doses were applied by attaching a piece of surgical tygon tubing to the syringe, inserting the tubing into the crop, and injecting the isotope solution. The syringe was weighed again after the oral dose was administered to determine the precise amount of isotope that was given. To administer the oral dose, each bird was removed from the box. At the same time, the feeder was removed and weighed to determine the half-hourly feed consumption. This procedure took approximately 20 seconds and likely did not significantly affect the collection of $^{14}CO_2$. Gas washing bottles were changed each half hour and immediately replaced with bottles containing fresh absorber. The weight of absorber was recorded and a sample taken for analysis on a scintillation counter as described by Tabiri (2002b). This allowed

calculation of the total radioactivity that was exhaled by the individual bird during each half hour. The % dose retained was calculated by using the following formula:

% Dose Retained = 100*[Total dose (DPM) – Total Exhaled (DPM)]/ Total Dose (DPM)

where DPM is disintegrations per minute of ^{14}C. Once the oxidation experiment was complete, the four birds were killed via cervical dislocation. The total of 16 birds removed on each sample day were dissected and the right femur removed. Bones from each bird were immediately analyzed by QCT using a Norland Stratec XCT[1] scanner. The procedure used has been reported previously (Saunders-Blades et al., 2003). Briefly, an initial longitudinal scan was performed on each bone. From this image, the midpoint of the bone was determined, and a cross sectional scan was performed at that point. The cross-sectional scan quantified the bone density and area of cortical, trabecular, and total bone of a 1 mm cross-sectional slice.

Statistical Analysis

Plateaus in % dose retained were initially determined using linear regression (SAS, 1999) with a non-significant slope ($P>0.10$) and a $CV<15\%$ considered a plateau. Differences between mean recoveries, densities and areas at different ages were analyzed using the pdiff option of the Proc GLM function of SAS (SAS, 1999). Correlations and regressions were performed using the Proc Corr and Proc Reg functions of SAS software respectively. Significance between means were considered significant at the $P < 0.05$ level. The affect of age on BRF, using BW as a covariate was determined using the Proc GLM function of SAS.

[1] XCT Research SA, Norland Corp., Fort Atkinson, Wisconsin, USA

Results and Discussion

The pattern of $^{14}CO_2$ retention is shown in Figure 2.1. Bicarbonate retention was shown to increase from 7 d to 21 d, then decrease to 42 d. The retention factors determined on days 7, 35 and 42 fall within the expected range, however the retention factors for days 0, 14, 21, and 28 (Table 2.2) were higher than expected based on experiments in broiler breeders (Tabiri et al., 2002a). Experiments in other animals have resulted in a variety of retention values, ranging from 14% in broiler breeders (Tabiri et al., 2002a), 21% in dogs (Downey et al., 1986), 22 to 26% in neonatal pigs (Benevenga et al., 1992), and from 6-48% in humans (Hoerr et al., 1989; el-Khouri et al., 1994). Although these values are not expected to be identical to those for broiler chickens, they provide a background from which expected values could be drawn.

The percent retention of bicarbonate on day 0 resulted in a high CV (18.4%); this was thought to be a result of the changing body composition of the bird during the early post-hatch period. At the day 0 sample day, chicks were not allowed to consume water prior to the experiment, because the trial was performed immediately after they arrived from the hatchery. Although water consumption was not measured, the chicks were observed to spend a large amount of time drinking. This, in combination with the water that was included with the isotope gavage, likely had an impact on the body composition of the bird. Assuming a 42 g chick with 50% DM, consumption of 1 additional g of water would increase the water mass in the chick's body by 4.5%. Since the bicarbonate pool size is the product of the concentration of bicarbonate and the volume of the pool, an increase in the volume of the pool would increase the chicks' capacity for bicarbonate retention, particularly since body bicarbonate is regulated via its concentration (Whittow, 2000). This may lead to an erroneously high retention factor; the exhalation of $Na^{14}CO_3$

was simply delayed by the changing pool size rather than lost to irreversible metabolic pathways, or slowly exchanging pools. For this reason, oxidation experiments in very young chicks should be avoided or involve monitoring the water intake of the chick throughout the experiment, and the use of a more concentrated isotope solution. Alternatively, experiments using young chicks may be performed without access to water other than that which is gavaged, in order to keep a relatively constant body water volume. Water consumption should not be a concern in older birds, as they do not enter the experiment in a dehydrated state, and they will likely not significantly affect the amount of body water though water consumption.

A quadratic relationship ($P<0.0001$, $R^2= 0.8882$) between bicarbonate retention and BW, from 7 to 42 days, was found (Figure 2.1). The data for day zero were excluded from the regression analysis because they were deemed unreliable (see above). The equation shown on Figure 2.1 can be used to predict the bicarbonate retention using the BW of the bird. The BRF appears to plateau at 35 days of age, so the average value for bicarbonate retention for 35 and 42 days of age (33.9% retention) can be used in place of the above equation. This equation was developed using birds fed a complete, commercial-type diet. The use of this equation assumes that the experimental diet has not significantly altered the physiological state of the bird, and that the dietary treatment does not affect the metabolic processes that result in the fixation or retention of bicarbonate. The relationship between bicarbonate retention and age was significant ($P<0.01$), while using BW as a covariate was not significant.

The relatively high retention factors found at 14, 21 and 28 d may be explained by the pattern of development of the chick. As broilers grow very rapidly from 0 to 42 days, the incorporation of bicarbonate into bone pools may contribute significantly to the

relatively low recovery of infused bicarbonate. Total bone cross-sectional area nearly doubled each week, from 10.72 mm^2 at 14 days to 22.09 mm^2 at 21 days and 41.35 mm^2 at 28 days (Table 2.3). Cortical bone area followed a similar trend with cross-sectional area of 5.39 mm^2 at 14 days, 12.84 mm^2 at 21 days and 23.10 mm^2 at 28 days. After 28 days, proportional bone growth was somewhat reduced, increasing by a factor of about 1.5. The growth in cross-sectional area is roughly proportional to the increase in body weight of the birds from day 7 to 42. Total and trabecular bone density, however, decreased after 28 days while cortical bone density continued to increase during this period. The continued increase in area and decrease in density suggest that the rate of bone deposition as a proportion of BW decreases after 28 days. Assuming the femur can be taken as representative of total body bone growth, this high growth rate of bone could partially account for the high bicarbonate retention factors found in 14 to 28 day old birds. The correlation ($r=0.50$, $P=0.0066$) between bicarbonate retention and total bone density shows that much of the variability in bicarbonate retention was a result of changing bone density (Table 2.4). This helps to explain the relatively high values for retention found in young, rapidly growing birds. The total bone density for the broilers in this trial was highest at 21 and 28 days. It should be recognized that the measurements of bone area and density used here were made at the midpoint of the femur, and assumes that this measurement is proportional to the development of the rest of the skeleton. Applegate and Lilburn (2002) have shown that the femur is more proportional to total bone development than the tibia. That study also showed that femur length reached a plateau at 35 days of age, and epiphyseal and diaphyseal ash reached a plateau at 21 d. This indicates that bone growth at the growth plates slows at around 3-5 weeks of age, about the same timeframe as the decrease in bicarbonate retention. This study did not quantify bone growth at the growth plates, but analyzed bone cross-sectional area. While there was no significant correlation between bone cross-sectional area and bicarbonate

retention, the proportional rate of growth decreased between 28 and 35 days of age, which corresponds to the drop in bicarbonate retention.

Studies by Poyart et al. (1975a; 1975b) in rats have shown that bone can act as a bicarbonate pool. These authors showed that 30-50% of the bone CO_2 stores are dissolved in water associated with the bone tissue, and can be exchanged with blood CO_2. The remaining 50-70% of bone CO_2 is associated with the crystalline structure of the bone, and will exchange with the aqueous portion at a very low rate. They also showed that the water associated with the bone declines with age (Poyart et al., 1975b). If this holds true in poultry, it could explain the rapid decrease in bicarbonate retention as birds' age past the period of most rapid bone development. In rats, 30% of the CO_2 in the blood that was partitioned to bone tissue was taken up by the bone (Poyart et al., 1975b). The amount of bicarbonate sequestered in the bone was limited by blood flow to the tissue rather than the exchange rate. As the fractional blood flow that reaches the skeleton in chickens is not known, the potential contribution of this slowly-exchanging pool cannot currently be accurately estimated. As bone cross-sectional area is increased more rapidly than BW from 14 to 28 days of age, as shown in Table 2.3, it would be expected that a higher proportion of the bicarbonate pool would be sequestered during this period than during periods of relatively slower bone development. As well, Poyart et al. (1975a; 1975b) showed that the proportion of water associated with bone decreases with age. Since the largest loss of bicarbonate associated with the bone is due to exchange with this water, a decrease in the water associated with the bone would result in a decrease in the proportion of bicarbonate that is sequestered.

The efficacy of bicarbonate retention trials in estimating the amount of bicarbonate liberated from substrate oxidation has been questioned (Hamel et al., 1993).

One of the main concerns of critics is that the bicarbonate infused into the blood stream does not have the same starting point as bicarbonate that arises from oxidation. That is, infused bicarbonate enters the plasma pool while bicarbonate from oxidation first enters the intracellular pool. The distribution of infused $Na^{14}CO_3$ throughout body tissues has been investigated in several studies. Hamel et al. (1993) showed in humans that there was disequilibrium between the labeled bicarbonate in the breath, and label incorporation into urea in the liver. This was taken as an indication that the CO_2 pool in the liver had a lower enrichment than the plasma pool. This may also be due to the use of arginine in the formation of urea, and may not be completely accurate as an indication of enrichment. Since, during amino acid oxidation studies, the labeled CO_2 production resulting from oxidation of a labeled substrate occurs within the cell, the amount of the label reincorporated into metabolic products may be underestimated if the infused $Na^{14}CO_3$ fails to equilibrate between plasma and cellular pools. Tomera et al. (1983), however, used perfused rat livers to show that bicarbonate retention does in fact represent the re-incorporation of CO_2 from substrate oxidation by the liver. The effect of other organs has not been studied. Since the pool in which the label appears in oxidation studies is not the plasma pool, as is the case with bicarbonate retention studies, failure of all body pools to reach the same enrichment within the time frame of the experiment would result in underestimation of the proportion of the label that is incorporated into metabolic products. This would lead to underestimation of the bicarbonate retention factor and the consequent underestimation of the rate of oxidation of the substrate. However, when CO_2 in the breath reaches a plateau during an oxidation study, it is assumed that isotopic steady state has been reached in all significant body pools.

There have been several different approaches by researchers attempting to account for the effect of CO_2 retention during substrate oxidation studies. Many authors

(Clugston and Garlick, 1983; Tomera et al., 1983; Hoerr et al., 1989) suggest that the only accurate method to account for CO_2 retention is to perform a retention study either concurrently (using ^{13}C labeled CO_2 and ^{14}C labeled substrate or vice versa) with the oxidation study, or to perform a retention study the previous day in the same individual under the same metabolic conditions. Other authors (Irving et al., 1983; Van Aerde et al., 1985; Benevenga et al., 1992; Tabiri et al., 2002a; 2002b) feel it is an adequate approach to determine average retention values for similar subjects under the same metabolic conditions, and apply those values to oxidation studies. The former approach is undoubtedly the more accurate, but involves several drawbacks for studies on growing animals. Firstly, the use of ^{13}C labeled bicarbonate requires the use of expensive equipment (e.g. mass spectrometer), as well as the relatively expensive isotope itself and an extensively trained staff. As well, as this study has shown, the bicarbonate retention factor changes rapidly with body weight and would require retention experiments to be performed between consecutive oxidation days. This approach effectively doubles the time and cost associated with the experiment. For these reasons many authors, particularly those studying animals, have accepted the latter approach. For broiler chicken oxidation studies, the regression equation described in Figure 2.1, with an R^2 value of 0.8882 is sufficiently accurate for body weights between 100 and 2700 g. Studies involving different types of chickens, or body weights outside the described range, require an additional study to determine the bicarbonate retention factor. The approach described by Wolfe (1992), in which a bicarbonate retention study is performed immediately prior to an oxidation study may be appropriate for younger birds, with whom changing pool volume may cause problems. This approach involves using a constant infusion of labeled bicarbonate to determine the retention of bicarbonate, followed immediately by an unprimed infusion of the indicator amino acid. The bicarbonate infusion must be calculated to result in approximately the same plasma $^{14}CO_2$

as the predicted oxidation of the labeled amino acid. Although this method would be more time consuming and expensive, it would result in more reliable data.

This experiment has provided factors to correct for bicarbonate retention in broilers weighing between 100 and 2700 g. The total bone density, as determined by QCT, was shown to account for 25% of the variation in bicarbonate retention. The results of this experiment require the assumption that the experimental diets do not significantly alter the fixation of bicarbonate or the bird's ability to sequester bicarbonate in body pools.

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Tables and Figures

Table 2.1. Ingredient and calculated nutrient composition of commercial type starter (0-21 d) and grower (22-42 d) diets

Ingredient:	Starter Diet Amount (%):	Grower Diet Amount (%):
Wheat	62.10	53.50
Corn	1.87	14.84
Soybean Meal	24.50	2.19
Canola Meal	1.60	0
Canola Oil	5.50	5.6
Dicalcium Phosphate	1.48	1.11
Limestone	1.30	1.40
Choline Chloride[1]	0.05	0.50
Vitamin/Mineral Premix[2]	0.05	0.50
Salt	0.397	0.35
D,L-Methionine	0.157	0.083
L-Lysine HCl	0.041	0.108
Zinc Bacitracin	0.06	0.05
Amprol	0.06	0.05
Avizyme 1302	0.05	0.05
Calculated NutrientComposition:		
Protein (%)	21.4	19.6
ME (kcal/kg)	3000	3097
Linoleic Acid (%)	1.74	2.02
Lysine (%)	1.02	0.968
Methionine (%)	0.46	0.367
Methionine + Cystine (%)	0.84	0.710
Calcium (%)	0.930	0.865
Available Phosphorus (%)	0.418	0.337

[a] Choline premix provided 1,000 mg per kg complete feed.
[b] Vitamin Premix provided the following (per kg of diet): Vitamin A, 10,000 IU; Vitamin D_3, 2,500 IU; Vitamin K, 2.0 mg; Pantothenic Acid, 14 mg; Riboflavin, 5.0 mg; Folacin, 0.80 mg, Niacin, 65 mg; Biotin, 1.8; Thiamine, 2.0 mg; Pyridoxine, 4.0 mg; Vitamin B_{12}, 0.015 mg; Vitamin E, 35 IU; I, 0.50 mg; Mn, 70 mg; Cu, 8.5 mg; Zn, 80 mg; Se, 0.10 mg; Fe, 100 mg.

Table 2.2. Weekly body weight, and bicarbonate retention in broilers age 0-42 d, fed a commercial type diet free choice[1,2]

Age (d)	n	Average BW (g)	Mean Bicarbonate Retention (% of total oral dose)	CV[3] For Bicarbonate Retention
0	4	46.6 g	42.2 bc	18.4
7	4	109.1 f	27.4 d	8.7
14	4	251.1 e	47.2 ab	4.3
21	4	529.5 d	56.0 ab	9.6
28	4	1029.9 c	62.9 a	4.2
35	4	1828.8 b	33.4 cd	7.0
42	4	2647.5 a	34.5 cd	13.9

[1] Values were obtained using a 4 h constant infusion protocol.
[2] All birds received a priming dose of 1.2 μCi and an oral dose of 1 μCi each ½ hour.
[3] Coefficient of variation for mean bicarbonate retention was calculated among birds at the same age.
a-g means within a column with different letters are significantly different

Table 2.3. Body weight, bone density, and bone area of the femur in broiler chicks from 0 to 42 days of age (mean ± standard error)

Age (d)	n	Bird Weight	Total Density[1]	Total Area[1]	Trabecular Density[1]	Trabecular Area[1]	Cortical Density[1]	Cortical Area[1]
		g	mg/cm^3	mm^2	mg/cm^3	mm^2	mg/cm^3	mm^2
0	16	N/A	292.1 ± 8.66^{e}	2.68 ± 0.08^{f}	167.6 ± 8.33^{a}	1.52 ± 0.06^{f}	564.7 ± 4.16^{f}	0.43 ± 0.04^{g}
7	16	109.6 ± 1.14^{f}	405.1 ± 6.98^{d}	5.55 ± 0.13^{f}	156.2 ± 4.71^{a}	2.29 ± 0.07^{ef}	678.6 ± 5.44^{e}	2.17 ± 0.08^{f}
14	16	251.5 ± 2.73^{e}	457.7 ± 12.24^{bc}	10.72 ± 0.39^{e}	111.1 ± 10.09^{bc}	3.79 ± 0.14^{e}	720.8 ± 9.20^{d}	5.39 ± 0.31^{e}
21	16	526.9 ± 9.74^{d}	540.1 ± 10.2^{a}	22.09 ± 0.51^{d}	123.9 ± 9.46^{b}	6.43 ± 0.29^{d}	778.3 ± 8.57^{c}	12.84 ± 0.39^{d}
28	16	1054.1 ± 11.59^{c}	530.4 ± 11.04^{a}	41.35 ± 1.38^{c}	121.8 ± 17.3^{b}	14.33 ± 0.74^{c}	808.6 ± 8.13^{b}	23.10 ± 0.69^{c}
35	16	1857.5 ± 25.72^{b}	482.6 ± 11.95^{b}	67.17 ± 1.27^{b}	107.7 ± 8.51^{b}	28.29 ± 0.97^{b}	814.9 ± 7.95^{b}	33.15 ± 0.73^{b}
42	16	2568.1 ± 50.7^{a}	452.0 ± 9.63^{c}	80.77 ± 2.44^{a}	91.2 ± 7.65^{c}	38.20 ± 1.71^{a}	840.5 ± 4.05^{a}	36.06 ± 0.96^{a}

[a-f] Means within a column with no common superscript are significantly different ($P<0.05$).

[1] Values determined using Quantitative Computed Tomography at the midpoint of the femur.

Table 2.4. Correlations between body weight, age, bicarbonate retention and bone traits of broilers[1]

	BW	Age	% Dose Retained	Total Density	Total Area	Trabecular Density	Trabecular Area	Cortical Density	Cortical Area
BW	1.00	0.96185[x] <0.0001[y] 96[z]	-0.1972 0.3144 28	0.05240 0.6121 96	0.98097 <0.0001 96	-0.34665 0.0005 96	0.97036 <0.0001 96	0.77820 <0.0001 96	0.95675 <0.0001 96
Age		1.00	0.02633 0.8942 28	0.57703 <0.0001 112	0.95051 <0.0001 112	-0.48454 <0.0001 112	0.90085 <0.0001 112	0.90217 <0.0001 112	0.96466 <0.0001 112
% Dose Retained			1.00	0.50117 0.0066 28	-0.16396 0.4045 28	0.16059 0.4143 28	-0.28017 0.1487 28	0.09823 0.6190 28	-0.04985 0.8011 28
Total Density				1.00	0.35479 0.0001 112	-0.16671 0.0789 112	0.21093 0.0256 112	0.81511 0.0001 112	0.47652 <0.0001 112
Total Area					1.00	-0.45049 <0.0001 112	0.98007 <0.0001 112	0.76576 <0.0001 112	0.98290 <0.0001 112
Trabecular Density						1.00	-0.43149 0.0001 112	-0.43941 0.0001 112	-0.44417 <0.0001 112
Trabecular Area							1.00	0.68555 <0.0001 112	0.92886 <0.0001 112
Cortical Density								1.00	0.81877 <0.0001 112
Cortical Area									1.00

[x] = correlation (r)
[y] = Probability (P)
[z] = Number of observations (N)
[1] Total bone, cortical and trabecular areas and densities were measured using quantitative computed tomography at the midpoint of the femur. Values were calculated across a 1 mm thick slice of bone.

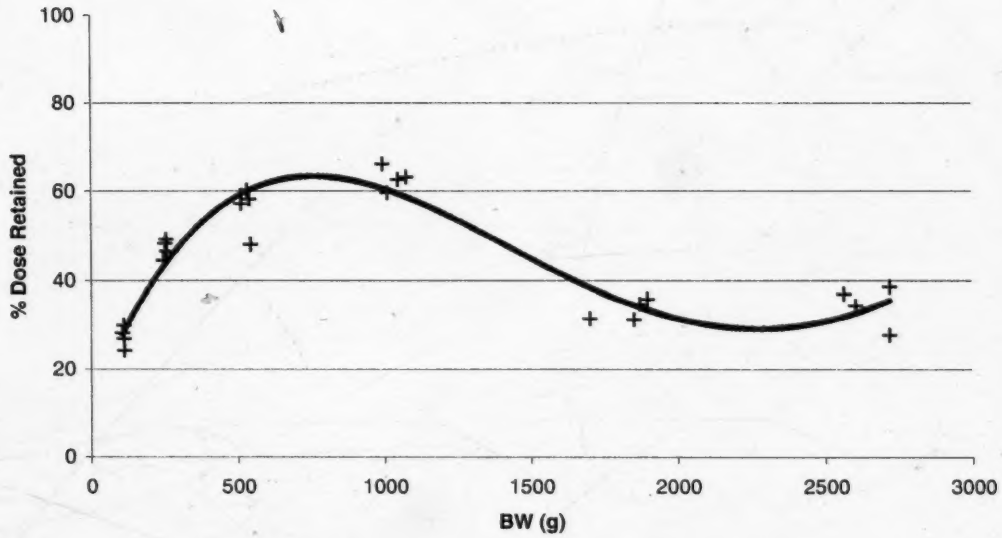

Figure 2.1. Bicarbonate retention, as a function of BW, for 7 to 42 day old broilers fed a commercial type diet free choice. The average BW for birds at 7, 14, 21, 28, 35, and 42 days of age were 109.1, 251.1, 529.5, 1029.9, 1828.8, and 2647.5 g respectively. The function $y = -5.5391*10^{-12}X^4 + 5.318986*10^{-8}X^3 - 1.5899*10^{-4}X^2 + 0.1587X + 13.3228$ describes the relationship between bicarbonate retention (y) and BW (x) with an $R^2 = 0.8882$. Birds were orally dosed with a prime of 1.2 μCi of $Na^{14}CO_2$ and 1 μCi of $Na^{14}CO_2$ per hour (in half hourly doses) for four hours and plateau retention values were calculated.

CHAPTER 3: ADAPTATION PERIOD AND PHENYLALANINE DOSE LEVELS

Introduction

The Indicator Amino Acid Oxidation (IAAO) method has been used in humans and animals to determine the requirements for indispensable amino acids and total nitrogen for maximum protein synthesis (Kim et al., 1983; Ball and Bayley, 1985; 1986; Zello et al., 1993; House et al., 1998; Coleman et al., 2003). Two approaches to the IAAO method have been used in various animals. The first is the bolus dose method, in which a large, single dose of the indicator is injected or fed, and the amount of labeled CO_2 that is exhaled over time is determined (Brookes et al., 1972). Once the level of labeled CO_2 that is exhaled plateaus, the percent of the bolus dose that is oxidized is calculated. It has been shown that this approach is not suitable for use in substrate oxidation studies (Issekutz et al., 1968). This is because the bolus dose method does not measure oxidation rate during isotopic equilibrium, and therefore does not accurately reflect the rate of oxidation of the substrate. The second is the constant infusion method, which measures the percent dose oxidized at isotopic steady state (House et al., 1998; Zello et al., 1993; Bertolo et al., 1998). This method usually involves surgery to insert a catheter in a peripheral vein to facilitate the infusion of isotope. A priming dose is injected via the catheter and the constant infusion ensues. The priming dose function is to decrease the time required to reach an isotopic steady state without affecting the plateau value that is obtained (Wolfe, 1992). Isotopic steady state is defined as the point when the rate of appearance of the isotope in the primary body pool is equal to the rate of disappearance (Wolfe, 1992). Appearance into the plasma pool, when using ^{14}C-phenylalanine (Phe), only occurs from two sources: 1) the infusion and 2) reappearance of ^{14}C-Phe that has left the primary pool and returned from peripheral pools during the

experimental period. Disappearance of free ^{14}C-Phe from the primary pool results from either fixation via protein synthesis, mixing with peripheral pools, or oxidation by catabolic enzymes in the liver. The appropriate priming and constant infusion doses that result in the rapid attainment of isotopic steady state are important for the accurate measurement of the rate of Phe oxidation in a limited timeframe.

Because the IAAO method measures the rate of catabolism of the indicator amino acid, time must be allowed for catabolic enzymes in the liver to adjust to the change in amino acid profile in order to obtain a stable value. The period required for birds to adapt to diets differing in the level of the test amino acid must be determined to ensure oxidation values are indicative of the test amino acid level. If birds are not adapted to the diets for the appropriate length of time, there will be little correlation between dietary amino acid profile and oxidation of the indicator. Adaptation periods in other types of amino acid studies, for example nitrogen balance studies, have been relatively long due to the need to allow all nitrogen pools to reach equilibrium. Studies in humans (Zello et al., 1990) and pigs (Ball et al., 2002) using the IAAO method have shown adaptation to occur within 2-3 days. This is likely because the adaptation in these studies only involves adjustment of catabolic enzyme levels and rates of protein turnover. Protein turnover likely plays a minor role, as the amount of most amino acids that arise from protein turnover are generally small in comparison to the amount arising from the diet (Harper, 1974). Since there is no long-term storage mechanism for free amino acids (Harper, 1974), the mechanism designed to conserve amino acids when deficient or eliminate excess amino acids, must respond rapidly to maintain homeostasis.

Most experimental protocols using the IAAO method at steady state involve surgical implantation of a catheter for the infusion of the indicator into the jugular vein

(Bertolo et al., 1998; House et al., 1998; Tabiri et al., 2002a; 2002b; Coleman et al., 2003). This method poses problems in broilers, as the surgical procedure imposes a stress on the animal that requires time to recover. This recovery period will result in reduced growth rate, and may not yield valid results in rapidly growing birds. In addition, the rapid growth of the bird poses a problem with maintaining catheters in the correct position. As the bird grows, the catheter, which is fixed to tissue in the neck, would most likely pull out of the jugular vein or shift into a sub-optimum position. Isotopic tracer protocols in broiler chickens, which employ a variation of the bolus dose method, have been developed which use an oral dose methodology (Ewing et al., 2001). Birds can be gavaged with two allotments of feed, given 2 hours apart, which contain the isotope indicator. This method has the advantages of oral dosing, but does not measure oxidation in an isotopic steady state. A method that combines the advantages of isotopic steady state and the convenience of oral dosing has been developed for use in human studies (Bross et al., 1998; Kriengsinyos et al., 2002), but has not been developed in poultry. The methods involved feeding several small meals, containing the indicator amino acid, during the experimental period. Meals are typically fed each hour in order to approximate a constant infusion protocol. This methodology was developed in order to apply the IAAO method to populations that are sensitive to invasive procedures, such as premature infants (Bross et al., 1998) and unhealthy patients (Bross et al., 2000; Courtney-Martin et al., 2002).

The current experiments were designed to determine some of the appropriate conditions for the use of the IAAO method in broilers. An oral dosing protocol, suitable for a primed constant infusion studies, was developed. Both the ratio of priming to oral dose required to rapidly achieve plateau, and the level of radioactivity required to provide sufficient counting accuracy, were determined. The period of adaptation that was

required to reach a new and stable level of oxidation of the indicator after a change in the level of the test amino acid was also determined.

Materials and Methods

Stock and management

All procedures were reviewed and approved by the University of Alberta Faculty Animal Policy and Welfare Committee, and were in accordance with Canadian Council on Animal Care guidelines (CCAC, 1993). Two groups of 20 male Ross 308 broilers were obtained from a commercial hatchery on the day of hatch. The groups were obtained several weeks apart, the first group being designated for Experiment 1 and the second group for Experiment 2. They were housed in a Petersime brooder battery, and allowed free access to feed and water at all times. Room temperature was maintained according to industry standard practices and adjusted according to behavioral changes in the birds. The lighting program used was 23:1 (L:D) for the duration of the experiments. In Experiment 1, a commercial-type broiler starter diet was fed from 0 to 21 days and a grower diet from 22 days to the 42 days (Table 2.1). Birds for Experiment 2 were fed the same commercial-type starter diet to 14 days, followed by experimental diets that are described in the next section.

Experimental Design

In Experiment 1, at 21 days of age, four birds were selected for the Phe dose trial that were within 5% of the mean BW of the flock. The birds were maintained on the commercial diet with ad libitum access to both feed and water. Different combinations of priming (2, 3, and 4 μCi/kg BW) and hourly oral dose (2.5, 3.5, 4.5 μCi/kg BW/h) were tested on two birds at a time. Experiments were performed on alternate days, allowing for processing of samples between oxidation days. Combinations of priming and oral dose that resulted in the most rapid plateau in oxidation rate were repeated in order to

obtain 4 birds for these levels. The experiment was performed over a 12-day period, with birds between 21 and 33 days of age.

The oxidation procedure and equipment used have been previously reported for broiler breeder pullets (Tabiri et al., 2002a; 2002b), with some notable changes that will briefly be described. Birds were removed from the Petersime brooder, weighed, and the priming dose gavaged using a piece of Tygon® tubing attached to a syringe. The free end of the tubing was placed directly into the crop of the bird, and the dose injected. The syringe was weighed before and after the gavaging of the isotope to accurately determine the amount of isotope each bird received. The first half-hourly oral dose was applied 15 minutes after the priming dose, and the birds placed in the oxidation chamber. Birds were removed from the chamber for approximately 20 seconds each half hour to apply an oral dose of ^{14}C-Phe. At the same time, the feed consumption of each bird was measured, and the gas-washing bottles were changed with bottles containing fresh absorber (Tabiri et al., 2002a). The absorber in the first two bottles was pooled, and a total weight recorded and a 7 ml sample taken. The third bottle was weighed separately, and a 7 ml sample taken as well. Sub-samples were weighed and counted for radioactivity on a liquid scintillation counter. In this way, the total radioactivity (DPM) was calculated for each half hour collection period. The third bottle was sampled separately in order to confirm that only a small portion of the radioactivity (i.e.<5%) was reaching the last bottle, thereby confirming that all radioactivity exhaled by the bird was accounted for. At the end of the 4 h collection period, birds were returned to the Petersime batteries. The percentage of the oral dose that was oxidized by the bird was calculated using the following equation.

% Dose oxidized = [(DPM recovered/BRF)/DPM gavaged]*100

where BRF is the bicarbonate retention factor, and DPM is the decay in disintegrations per minute (DPM) of ^{14}C as determined by scintillation counting. The BRF was calculated using BW data as described previously (Chapter 2). This value was calculated for each half hourly sample collection and plotted over time. Plateau in percent dose oxidized was defined as the time point where all subsequent points on the line had non-significant slope and a CV<15%. Data for this experiment was analyzed between oxidation days, and time to plateau was calculated. Combinations of priming and hourly oral dose were initially tested on two birds. When time to plateau was low, the combination was tested on an additional two birds, as was the case for the 2 μCi/kg BW and 3.5 μCi/kg BW/hour, 3 μCi/kg BW and 3.5 μCi/kg BW, and 4 μCi/kg BW and 4.5 μCi/kg BW/hour priming and hourly oral dose respectively.

Experiment 2 was designed to determine the length of time required for broilers to metabolically adapt to diets differing in the test amino acid level. Two test diets were formulated (Table 3.1). These diets were pelleted, purified, complete diets (except for Lys) that were identical except for lysine, glutamate and starch levels. Diet 1 contained 5 g lysine/kg diet while diet 2 contained 13 g lysine/kg diet, representing 50% and 130% of the NRC requirements for lysine (NRC, 1994) respectively. Glutamate was added to keep the diets isonitrogenous, and corn oil to keep diets isocaloric, both at the expense of cornstarch.

At 14 days of age, two groups of four birds were removed from the previously described population and individually housed. Group 1 was initially fed Diet 1 for 7 days. On 7 d, an oxidation study was performed as described above, using the optimum priming and oral dose combination of ^{14}C-Phe determined in Experiment 1. After the

oxidation experiment was completed, the birds were returned to their cages, and the diet was changed to Diet 2 for the remainder of the experiment. Oxidations were performed on d 8, 9, 10 and 14 in order to determine the time required to reach a stable level of phenylalanine oxidation. The second group of birds underwent the same schedule, but was fed Diet 2 first for 7 d, and was switched to Diet 1 after the first oxidation. Breath samples were collected and the oxidation rate of ^{14}C-phe was determined. Data were analyzed to determine the length of time required to attain a new and statistically stable level of ^{14}C-phe oxidation.

Statistical analysis

Plateau in % dose oxidized was defined as points on the line with a non-significant slope, as determined by Proc Reg of SAS (1999) software ($P>0.10$) and having a CV<15%. The results of the adaptation were analyzed using the Proc Mixed procedure of SAS (1999) with bird as the subject, in order to account for the repeated observations. Differences between oxidation rates on different days were separated using the pdiff option of the Proc Mixed function of SAS software. Differences between means were considered significant at $P<0.05$.

Results and Discussion

In Experiment 1, two of the three treatments that were repeated resulted in one bird that did not reach plateau. The results showed that birds receiving a priming dose of 3 μCi/kg BW and an oral dose of 3.5 μCi/kg BW/hour reached plateau between 30 and 120 min into the experiment (Table 3.2). These results were the most consistent, and allowed calculation of percent dose oxidized over a 2.5 to 4 hour period. A longer period of time in isotopic steady state allows more accurate calculation of the % dose oxidized of the indicator amino acid, which is the value of interest in IAAO experiments. A ratio of prime to hourly oral dose of 0.87 was found to be most appropriate for IAAO studies in these birds. The amount of the prime and oral dose can be adjusted according to the amount of expired $^{14}CO_2$ required for accurate counting on the scintillation counter, provided that the ratio is maintained. The purpose of the priming dose is to rapidly bring the body pool levels of the labeled amino acid up to the level that would eventually be achieved by constant infusion of the isotope (Wolfe, 1992). For this reason, the level of the prime relative to the level of the oral dose is more important than the numerical level of either dose.

Tabiri et al. (2002b) showed an appropriate priming to oral dose ratio tested in broiler breeders to be 1.6, which was almost twice the value found in this study. The discrepancy between these results shows that the conditions required to perform IAAO studies in birds of the same species that are of different ages are not constant. The birds used in this experiment were between 21 and 37 days of age, resulting in values that are most likely applicable to broilers at all ages of interest (typically up to 6 weeks of age). It is expected that the prime to oral dose ratio would change with bird age, as illustrated by the following equation from Wolfe (1992).

P/F = Pool size/Rate of appearance

Where P is the priming dose, F in the constant infusion dose, the pool size refers to the indicator amino acid, and the rate of appearance is that of the indicator amino acid from all sources. The bird's Phe pool will increase in size as the bird grows, which is accounted for in part by the application of each dose on a kg BW basis. This will likely not completely compensate for changes in pool size, since the proportions of metabolically active tissue and bone in the bird will change over time. As well, the rate of appearance will change with age, as the birds feed consumption will increase and turnover rates of protein in tissues will likely decrease with increasing age. The degree of change would likely be small during the age range of interest, but may be significantly influenced by the large differences in age or feed restriction involved in breeder management.

Figures 3.1 and 3.2 represent the results for Experiment 2. The oxidation rates for day 0 represent the initial diet (13 g Lys/kg diet in Figure 3.1, and 5 g Lys/kg diet in Figure 3.2), while the remaining points represent the second diet (5 g Lys/kg diet Lys in Figure 3.1, and 13 g Lys/kg diet in Figure 3.2). Adaptation from a diet with excess lysine required 2 days to achieve a new and stable rate of oxidation (Figure 3.1). However, only 1 day was required to achieve a new, statistically different rate of oxidation when the diet was changed from limiting to excess. These results closely resemble those found by Zello et al. (1990), Hoerr et al. (1993), Motil et al. (1994), and Thorpe et al. (1999) in humans and Ball et al. (2002) in swine.

Several studies using various isotopic tracers have investigated the effect of protein intake on the flux rate and oxidation of the labeled amino acid (Hoerr et al., 1993; Motil et al., 1994; Thorpe et al., 1999). Hoerr et al. (1993) adapted young men to diets

that were deficient in protein (0.1 g/kg BW/day) for 9 days, and estimated flux and oxidation rates using several isotopically labeled Lys and Leu tracers. The protein level was then increased to an excess level (1.5g/kg BW/day), where flux and oxidation rates were determined again. The researchers concluded that 1 d of high protein intake was inadequate to restore flux and oxidation rates to their baseline levels. In addition, they showed that protein synthesis was markedly reduced upon feeding of the low protein diet, while protein breakdown was only marginally reduced. Upon feeding of the high protein diet, protein synthesis increased rapidly while protein breakdown decreased further. This suggests that any carry-over effect from the low protein diet was due to the adaptive response designed to conserve amino acids. Motil et al. (1994) showed similar results in adult women. That study showed no change in protein turnover regardless of the change in protein level (3.5-fold difference between diets), probably due to the fact that all diets were formulated to meet the requirements of the subjects. Motil et al. (1994) suggested that 3 days were required for equilibration of oxidation rates. Thorpe et al. (1999) showed that at least 2 days were required for adaptation in humans to diets differing in protein content when flux measurements are incorporated into estimates of oxidation. They also suggested that no prior adaptation is required if oxidation is estimated by appearance of label in breath alone.

Metabolic adaptation to the changing level of the test amino acid, in this case Lys, is thought to mainly involve the regulation of catabolic enzymes in the liver (Motil et al., 1994). Once the enzymes responsible for the regulation of plasma Lys have adjusted to the new level of the amino acid in the diet, and the enzymes that regulate the other amino acids have adjusted appropriately, the bird should achieve a new and stable rate of oxidation of the indicator. Since there is no accepted mechanism of storage of amino acids (Harper, 1974), and the regulation of catabolic enzymes is expected to be

rapid in order to maintain homeostasis, it was expected that adaptation would occur in 1 d for both treatments. There are several possible explanations for the two-day adaptation that was observed when dietary Lys was reduced from excess to deficient. Firstly, protein turnover in the metabolically active tissues of the bird may require time to adjust. If turnover is high during periods of adequate amino acid intake, the amino acids from body tissue may buffer the deficiency of Lys in the diet for a brief time. Alternately, the breakdown of regulatory enzymes and other proteins with varying life spans could act as a buffer for the Lys deficiency in the short term. Bos et al. (2003) have shown in pigs that protein turnover of intestinal tissue after a bolus meal can result in a net release of absorbed Lys for at least 8 h after the meal. This short-term storage of amino acids may help explain the results seen here.

In contrast to the results presented by Hoerr et al. (1993), this study shows that adaptation of catabolic enzymes, as measured by stabilization of oxidation rate of the indicator amino acid, when an adequate diet was fed after a period of deficiency, adaptation occurred in one day. Flux and turnover rates were not measured in this experiment, so a complete comparison cannot be made, although the carryover effect of the low protein diet seen in that trial was not evident from the results presented here.

Since both Experiments 1 and 2 required several oxidation experiments within each bird on the same diet, an estimate of the variation within and among birds can be made (Table 3.3). Results presented for Experiment 1 only include data from birds that reached plateau, and data for Experiment 2 only includes those points that were performed within each bird after a new steady rate of oxidation of ^{14}C-Phe was reached. The SEM within birds for the excess Lys diet in Experiment 2 shows rather high variation within birds (1.94 to 2.60), as with low variation between birds (0.99). Results from the

Lys deficient diet in Experiment 2 showed relatively low variation within-birds (0.43 to 2.02 SEM) and between-birds (0.57). Experiment 1, however, did not result in variation comparable to that in Diet 2 in Experiment 2. This complete commercial diet showed within bird variation between 0.42 and 3.16 SEM, and between-bird variation of 0.99 SEM. The results from Experiment 2 suggest that % dose oxidized values obtained from diets deficient in the test amino acid are more precise than those obtained by feeding a diet sufficient in the test amino acid. These results are of interest because both sufficient and deficient diets must be fed during IAAO studies in to determine the requirement for the test amino acid. The amount of this variation that is due to the changing age of the bird is not known.

In summary, the priming dose to hourly oral dose ratio that was found to be the most consistent, and resulted in the most rapid achievement of plateau, was 0.87. The levels of 3 μCi/kg BW (priming dose) and 3.5 μCi/kg BW/hour (hourly oral dose) also resulted in high enough counts in the oxidation system used here to determine differences between treatments. The results of Experiment 2 suggest an adaptation period of 1 day when the diet in changed from deficient to sufficient, and 2 days when the diet is changed from sufficient to deficient; it is recommended that a 2 day minimum adaptation period be allowed in all cases to minimize the possibility of inadequate adaptation.

The data obtained in these experiments suggest that within-bird variation is as high as between-bird variation when multiple measurements are used to determine the rate of oxidation for a given diet, which may be a result of the period of time over which the trials were performed. Changes in BW and body composition over the duration of the experiment in a growing animal may affect the rate of oxidation of the indicator. One can conclude that, although experiments performed to determine the requirements of a single

bird would be more accurate than those using different birds for each data point to determine the requirement for a population, requirements could be determined over a very short time period if multiple measurements for each bird were employed. That is, if a set of birds at a given age and BW were used to determine the rate of oxidation at one level of dietary Lys, and multiple measurements were performed on each bird, a different set of birds could be used for each point on the graph. This would allow the precise determination of amino acid requirements over a two or three day period (eg. 7-10 days of age). The former approach also has the advantage of supplying information on the variability of the requirement within the population, and will allow the calculation of 95% confidence intervals for the population. The latter approach has the advantage of determining requirements for amino acids over a very small age range, which was previously not possible.

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Tables and Figures

Table 3.1. Ingredients and calculated composition of purified, pelleted grower diets (Experiment 2)

Ingredient:	Diet 1:	Diet 2:
	g/kg	
Corn Starch	576.1	571.4
Corn Oil	100.0	100.0
Solka Floc	26.0	26.0
Limestone	10.0	10.0
Dicalcium Phosphate	25.0	25.0
Salt	4.6	4.6
Vitamin/Mineral Premix[1] (g/kg)	22.0	22.0
Ethoxyquin	10.0	10.0
Amino Acids		
Lysine[2]	6.33 (5.00)	16.46 (13.00)
Methionine[2]	4.33 (4.24)	4.33 (4.24)
Cystine	4.54	4.54
Threonine	8.22	8.22
Phenylalanine	8.00	8.00
Tyrosine	7.00	7.00
Valine	10.00	10.00
Leucine	13.33	13.33
Isoleucine	8.89	8.89
Arginine	13.44	13.44
Serine	6.45	6.45
Glycine	6.44	6.44
Histidine	3.89	3.89
Tryptophan	2.00	2.00
Glutamate	125.42	120.00
Calculated Composition		
ME (kcal/kg)	3799	3799
CP(%)	19.5	19.5
Linoleic Acid (%)	5.4	5.4
Lysine (%)	0.5	1.3

[1] Vitamin Mineral Premix provided the following per kg of diet: Vitamin A, 10,000 IU; Vitamin D_3, 2,500 IU; Vitamin K, 2.0 mg; Pantothenic Acid, 14 mg; Riboflavin, 5.0 mg; Folacin, 0.80 mg; Biotin, 1.8 mg; Niacin, 65 mg; Thiamine, 2.0 mg; Pyridoxine, 4.0 mg; Vitamin B_{12}, 0.015 mg; Vitamin E, 35 IU; Mn, 70 mg; Cu, 8.5 mg; Zn, 80 mg; Se, 0.10 mg; Fe, 100 mg; Choline, 1,000 mg.

[2] Lysine and methionine were added as feed grade ingredients. Numbers in brackets refer to the amount of those amino acids added.

Table 3.2. Priming dose and hourly dose of ^{14}C-phe, and resulting time to plateau of birds fed a commercial diet

Treatment #	Priming Dose (μCi/kg BW)	Oral Dose (μCi/kg BW/hour)	N[3]	Ratio[1]	Time to Plateau (min)[2]	Average % Dose Oxidized
1	2	2.5	2	0.80	120-150	6.52
2	2	3.5	3	0.57	60-90	7.15
3	3	2.5	2	1.20	60-90	14.09
4	3	3.5	4	0.87	30-120	10.45
5	3	4.5	2	0.67	60	13.09
6	4	2.5	2	1.60	120	10.82
7	4	3.5	2	1.14	150	11.40
8	4	4.5	3	0.89	90-150	7.07

[1] Prime to hourly oral dose ratio.
[2] Points on the plateau haved a CV<15%. The experiment was 240 min in duration, therefore in order to have a minimum of 3 points on the plateau, a maximum of 150 minutes to reach plateau was allowed.
[3] All treatments were performed on 2 birds, except treatments 2, 4, and 8, which had 4 birds. N describes the number of birds that reached plateau.

Table 3.3. Mean % ^{14}C-phe dose oxidized and SEM for points on different oxidation days in Experiments 1 and 2

	n	Mean % dose Oxidized	SEM
Experiment 1: [2]			
Bird #:	**Commercial-Type Feed**		
1	6	21.44	3.16
2	3	18.57	1.34
3	4	14.93	1.24
4	6	16.90	0.42
Mean		17.96	0.99[1]
Experiment 2:[3]			
Bird #:	**Pelleted, Purified Feed- Excess of Lysine**		
1	3	5.99	1.40
2	3	9.74	2.60
3	3	9.21	1.96
4		9.78	1.94
Mean		8.69	0.99[1]
Bird #:	**Pelleted, Purified Feed- Deficient in Lysine**		
1	4	13.02	0.43
2	4	12.09	0.97
3	4	14.01	2.02
4	4	12.68	1.00
Mean		12.95	0.57[1]

[1] SEM values calculated using mean percent dose oxidized values for each bird.
[2] Experiment 1 was designed to test different combinations of priming and oral dose levels on the time required to reach plateau. A complete commercial type diet was fed to all birds. Variation data was calculated using only birds that reached plateau.
[3] Experiment 2 was designed to determine the time required to reach a steady rate of indicator amino acid oxidation when the level of test amino acid was changed. Both an adequate and a deficient purified diet were used. Variation data was calculated using only points that were obtained after a steady rate of oxidation was reached.

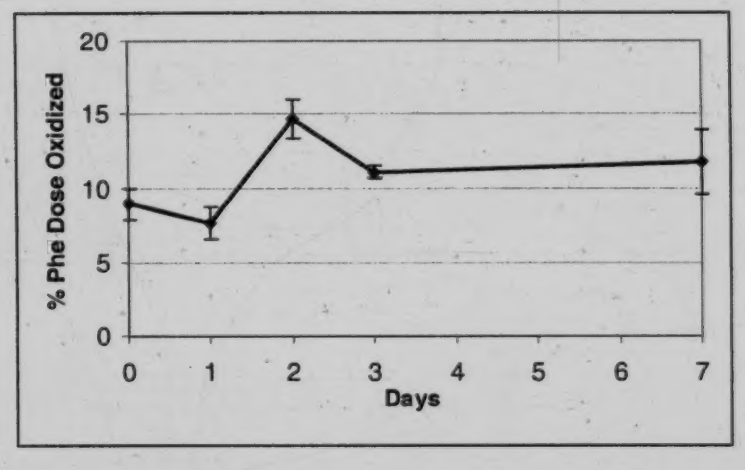

Figure 3.1: Adaptation in % ^{14}C-Phe dose oxidized from a diet containing 13 g lys/kg diet (d 0) to a diet containing 5 g lys/kg diet (d 1-7). Each data point represents the mean oxidation rate of ^{14}C-phenylalanine averaged over 4 male broilers.

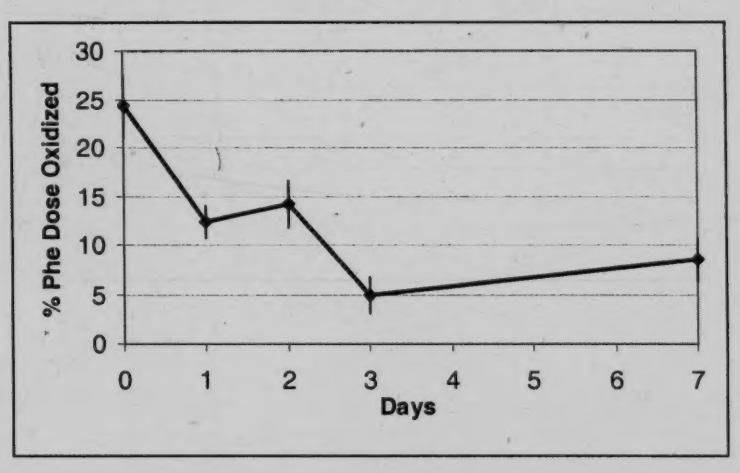

Figure 3.2: Adaptation in % ^{14}C-Phe dose from a diet containing 5 g lys/kg diet (d 0) to a diet containing 13 g lys/kg diet (d 1-7). Each data point represents the mean oxidation rate of ^{14}C-phenylalanine averaged over 4 male broilers.

CHAPTER 4: SLOPE RATIO AND IAAO AVAILABILITY

Introduction

Accurate determination of the digestibility or availability of amino acids in feedstuffs for poultry is essential for cost-effective formulation of rations. Current NRC (1994) requirements for poultry are presented as total amino acids in the feed, which overlooks the ability of the bird to digest and absorb those amino acids. NRC requirements for swine (NRC, 1998) are currently defined in terms of adjusted ileal digestibility, which allows for more precision in feed formulation, provided the true ileal digestibility of the feedstuffs are known. The determination of poultry requirements for available amino acids for protein synthesis, as described by Coleman et al. (2003), will allow greater precision in diet formulation than either of total or digestible amino acids only if the availability of amino acids in feedstuffs are known. Availability is similar to digestibility, in that it compensates for indigestible amino acids, but further accounts for amino acids that are absorbed in a form that is not metabolically available to the bird (Sibbald, 1987). There is currently only one method that has been accepted to determine the availability of amino acids for protein synthesis. The slope ratio method, described by Batterham et al. (1979; 1984; 1986) and van Barneveld et al. (1994a; 1994b; 1994c) uses diets containing graded levels of the test amino acid in purified form, or via the test ingredient, and determines the availability through linear responses in parameters such as amino acid accretion, feed conversion, and growth. This method is time consuming, requires a large number of animals, and can be very costly.

Recently, the Indicator Amino Acid Oxidation (IAAO) method has been applied in poultry to determine the requirements for available amino acids (Tabiri et al., 2002a; 2002b; Coleman et al., 2003). These studies in the development of the IAAO method for use in chickens have shown that when all other amino acids are present in excess, the rate of oxidation of the indicator amino acid is affected in a linear fashion by increases in the level of the test amino acid below the requirement. When diets are deficient in the test amino acid, increases in that amino acid cause a linear decrease in oxidation of the indicator amino acid. This is due to the linear increase in protein synthesis allowed by the additional dietary test amino acid. Since this response is linear, the IAAO technique may be suitable for use in a slope ratio assay. As well, the use of the IAAO technique would allow the determination of amino acid availability within a single bird, allowing the variation in a population for the ability to utilize amino acids to be determined.

The experiments presented here are designed to test the validity the IAAO method for use in determining the metabolic availability of lysine in poultry feedstuffs. A trial was performed using the traditional slope ratio method, as described by Batterham (1979; 1984; 1986) to determine the availability of lysine in a sample of peas for both male and female broilers. Two additional experiments were performed to determine the availability of lysine in the same sample of peas using the IAAO method. Subsequently, some of the peas were heat-treated to reduce the lysine availability. The availability of lysine in cooked peas, and the cooked peas with lysine supplemented to the initial level, as lysine- HCl, was tested.

Materials and Methods

All procedures were reviewed and approved by the University of Alberta Faculty Animal Policy and Welfare Committee, and were in accordance with the Canadian Council on Animal Care guidelines (CCAC, 1993). In Experiment 1, two hundred male and two hundred female Ross 308 chicks were purchased from a commercial hatchery. The birds were placed in rearing cages[2] at a density of 10 birds per cage with 318 cm^2 per bird. All birds were fed a commercial-type starter diet from hatch to 20 days of age. Water was provided ad libitum for the duration of the trial. On day 20, the birds were weighed, and the 28 males and 28 females that were closest to the mean body weight for each sex were randomly assigned to individual cages. The seven dietary treatments (Tables 4.1 and 4.2) were randomly assigned to 4 males and 4 females. A further 6 male and 6 female birds were killed by cervical dislocation in order to estimate the initial body composition of the test birds. Lysine composition of birds and diets were determined through HPLC analysis (Sedgwick et al., 1991)

The seven diets in Experiment 1 were formulated to be isonitrogenous, isoenergetic and complete except for lysine. A basal diet was formulated to contain 5.5 g/kg diet lysine (55% of the NRC requirement). Diet 1 was the basal diet with cornstarch, and each of the other 6 diets were produced by adding either peas or lysine at the expense of cornstarch. Diets were kept isonitrogenous by adding crystalline glutamate. Corn oil was added to diets in order to maintain equal energy levels in all diets.

On day 20, prior to feeding the test diet, feed was withdrawn from birds for 8 hours to allow emptying of the digestive tract. Birds were then weighed, and the experimental diets provided. Birds had ad libitum access to feed and water. On day 30,

[2] Specht Canada Inc., Stony Plain, Alberta Canada.

feed was again withdrawn in order to facilitate digestive tract clearance, and then weighed. Feed intake was recorded, birds were killed by cervical dislocation and the carcasses frozen at –20 degrees C. Feed conversion and BW gain data were calculated.

After the birds were frozen, carcass lysine content was measured by HPLC analysis (Sedgwick et al., 1991). Due to the likelihood of lysine binding to other compounds in the Maillard reaction during heating, birds were not pressure-cooked, as is a standard procedure (Renema et al., 1999). Rather, frozen birds were first cut into smaller pieces with a band saw, and then forced through a meat grinder. A representative sample was taken, weighed, and freeze-dried. Dry matter was determined through weight difference of the samples before and after freeze-drying. The dried samples were then homogenized further in a coffee grinder prior to analysis. The carcass composition samples were analyzed for amino acid content, via HPLC, in order to determine lysine accretion (Sedgwick et al., 1991). Accretion was calculated by determining the difference between initial lysine content (initial BW multiplied by the lysine content of the birds killed at the beginning of the experiment) and the final lysine content.

In Experiment 2, twenty male Ross 308 chicks were purchased from a commercial hatchery. The chicks were placed in a Petersime battery brooder and provided a commercial starter diet and water ad libitum from hatch to 17 days of age. On day 17, the four birds closest to the mean BW of the population were selected and individually housed in the Petersime battery.

Diets for the IAAO availability trial were formulated to be isoenergetic, isonitrogenous and complete except for lysine (Tables 4.3 and 4.4). Similar to the procedure in Experiment 1, glutamate was used to maintain nitrogen levels in all diets

and corn oil was used to maintain energy levels. Phenylalanine levels were kept constant by adding crystalline phenylalanine to diets that did not contain peas. This was done to maintain a constant phenylalanine pool size and prevent variable dilution of the tracer. The availability of phenylalanine was assumed to be similar to that of lysine determined in Experiment 1. This assumption was based on the similar values for digestible phenylalanine and lysine in peas according to the swine NRC guide (1998). A basal diet was formulated to contain 5.5 mg/kg lysine (Diet 1). Other experimental diets were created by adding either pure lysine or peas, at the expense of cornstarch, to create diets ranging from 5.5 to 8.5 mg lysine per kg diet. The order in which dietary treatments were fed was randomly assigned.

Diets were fed for 2 days prior to the experimental day to allow metabolic adaptation to the new level of dietary lysine, as determined in Chapter 3. The procedure for the oxidation experiment has been described previously (Tabiri et al., 2002a; 2002b; Coleman et al., 2003), with some adjustments made for this experiment. Briefly, the birds were allowed ad libitum access to feed and water for the duration of the trial. Birds were placed in the polycarbonate oxidation chamber and were gavaged with a priming dose of 3 μCi per kg BW of 1-[L]^{14}C-phenylalanine (the indicator amino acid), followed after 15 min by the first of eight half hourly oral doses, totaling 3.5 μCi/kg BW/h. This has previously been determined to be the optimum priming and half hourly oral dose for broilers, as described in Chapter 3. The birds were enclosed in the chambers, and the pump started. The vacuum pump drew air out of the chamber, through a series of three gas-washing bottles, and out of the room. The gas-washing bottles contained a mixture of monoethanolamine and 2-methoxyethanol (1:2, vol:vol) as a CO_2 absorber, and were sampled each half hour at the same time the birds were removed from the chambers to

apply the oral dose. The rate of oxidation of the indicator amino acid was determined by the following equation:

% dose oxidized = [(Total DPM recovered/BRF)/Total DPM gavaged]*100

where BRF is the bicarbonate retention factor. The BRF was estimated using BW, as described in Chapter 2. The % dose oxidized was determined for each half hour sample period and graphed over time. The plateau in % dose oxidized was determined as described in Chapters 2 and 3. After the 4-hour oxidation experiment was completed, the birds were returned to their cages and the next diet was provided. Birds received on each test diet for a minimum of 48 hours, including the duration of the oxidation.

Diets 1 through 4 were designed to establish the linearity of the oxidation of the indicator amino acid to increasing levels of available lysine in the diet, where lysine was increased by increasing the addition of lysine-HCl. Diets 5, 6, and 7 were designed to test the availability of lysine in raw peas, cooked peas and cooked peas plus lysine-HCl back to the original level of available lysine, respectively. The peas used in this experiment were all from a single batch, and were the same as those used in Experiment 1. A portion of the peas was placed in a convection oven at 165 C for 4 hours in order to cause the Maillard reaction and reduce the availability of lysine (van Barneveld et al., 1994c).

The results of Experiment 2, as well as observations made during the experiment, indicated that the birds used were agitated by the environmental conditions of the experiment. This is believed to have affected the metabolic rate of the birds, and therefore, clearance of $^{14}CO_2$ from the birds system. The birds in this experiment were not adapted to the chambers as recommended by Tabiri et al. (2002a). Therefore,

Experiment 3 was conducted to make minor adjustments to the method described for Experiment 2. The dietary treatments and experimental procedure were identical to those in Experiment 2, with a few minor changes. In this experiment, eight birds (four males and four females) were obtained from a mixed-sex flock at 14 days of age. The birds were reared in the oxidation chambers from 14 days to the end of the experiment. Oxidations were performed every second day, beginning on day 19. The purpose of housing the birds in the chambers was to allow them to become accustomed to the novel environment. In addition, the pumps used to move the air through the chambers were turned on each day for two hours in order to allow the birds to adjust to the noise level. The birds were also handled frequently and gavaged with tap water to accustom them for the oxidation procedure. The birds in Experiment 3 did not show the same agitated behavior as those in Experiment 2.

The diets were fed in random order, with all birds receiving the same diet on the same day. The diets were identical to those used in Experiment 2 (Table 4.2), but were mixed at a later time. Thus, lysine content of the two batches were analyzed separately and presented in Tables 4.3 and 4.5. At the end of the trial, each of the eight birds where dissected to determine gender.

Statistical Analysis

In Experiments 2 and 3, plateaus in percent of L-[1-^{14}C]phenylalanine dose oxidized were determined using Proc Reg, where a non-significant slope ($P>0.10$) and a C.V. less than 15% were considered to be on plateau.

Regression equations in Experiments 1, 2 and 3 were determined using the Proc Reg procedure of SAS software (Version 8, SAS 1999). When linear regressions were not significant ($P<0.05$) availability was not calculated. If the response to free lysine was not linear, the availability was not calculated for any other treatment. Differences in feed consumption in Experiment 1, and differences between means in Experiment 3 were analyzed using PROC GLM of SAS software, with means separation performed using the PDIFF option. Differences between slopes and intercepts of regression lines in Experiment 1 were analyzed using Prism software, with differences between slopes and intercepts considered significant at $P<0.05$.

Results and Discussion

The initial mean BW (20 d of age) of the female and male Ross 308 birds in Experiment 1 was 592 ± 3.5 g and 609 ± 6.2 g, respectively. Growth response over the 10-day period was linear for both male and female birds when free lysine was added, but was not linear when peas were used as the source of lysine (Tables 4.6 and 4.7). This is not surprising, because feed consumption was not equal for all groups (data not shown). Feed conversion data showed linear decreases in response to increased lysine in both male and female birds regardless of the source of lysine. A comparison of the slopes between the free lysine and pea diets for the feed conversion response curves showed the availability of lysine to be 70.3% (Figure 4.1) and 61.4% (Figure 4.2) for male and female birds respectively. Lysine accretion data also showed linear increases in response to increased dietary lysine regardless of sex or source of lysine. The availability values determined for male and female birds, using lysine accretion data, were 70.1% (Figure 4.3) and 55.7% (Figure 4.4) respectively. The R^2 and P values obtained through the

linear regression procedure suggest that the availability determined using feed conversion for male birds was more precise whereas availability using lysine accretion data was more precise for female birds.

Analysis of differences between the response curves showed no significant difference between males and females in their feed conversion or lysine accretion response to addition of lysine as lysine HCl or peas. This allows the data for males and females to be pooled and used to calculate availability. The pooled availability of lysine in the pea sample was 57.1% using lysine accretion data and 64.3% using feed conversion data. Intercepts were not different between males and females for feed conversion response, while there was a significant difference between the intercepts for lysine accretion. Females had a significantly higher lysine accretion at zero lysine interval. This indicates that the female birds utilized the basal diet more efficiently for lysine accretion than males.

Experiment 2 was performed in order to validate the IAAO method for the determination of metabolically available amino acids in feedstuffs. The oxidation rates found in Experiment 2 showed that oxidation of the indicator varied dramatically between birds for each diet (Table 4.8). Oxidation of the indicator for the basal diet (Diet 1) for Bird 4 was three times as high as for Bird 2. It was expected that the baseline level of L-[1-^{14}C]phenylalanine oxidation would be similar between birds at a similar age and BW. Regression equations that were generated in Experiment 2 similarly did not follow the expected pattern (Table 4.9). It has been well established that the oxidation of L-[1-^{14}C]phenylalanine decreases in response to addition of the limiting amino acid to the diet for chickens (Coleman et al., 2003), pigs (Bertolo et al., 1998) and humans (Zello et al., 1995). The regression equations in Table 4.6 show that addition of lysine in the form of

peas increased oxidation of the indicator for Birds 1 and 2, and addition of lysine as lysine-HCl increased oxidation of the indicator for Bird 2. These results are shown in Table 4.9 as the positive slope on the regression lines. Birds 3 and 4 followed the expected pattern, and analysis of the slopes revealed availability values within the expected range, as determined by the slope ratio experiment.

The results for Birds 1 and 2 may be explained through the behavior of the birds during the experimental period. Observations during the first and second oxidation days showed that these two birds exhibited stress behavior, likely due to the novel environment in the chamber. As the experiment progressed, the birds became calmer. Birds 3 and 4 did not show a similar discomfort in the oxidation chambers. Studies on bicarbonate retention have shown that high metabolic rate affects the amount of labeled CO_2 that is exhaled by the animal (Barstow et al., 1990; Van Aerde et al., 1985). In effect, higher metabolic rate results in a more complete collection of the label than that seen in animals that are at rest, resulting in data points within a bird being artificially inflated. Although metabolic rate was not measured in this experiment, the nervous behavior exhibited by Birds 1 and 2 may have affected the amount of label that was collected, and compromised the accuracy of the BRF that was used to compensate for retained label. If this were the case, those data points that were obtained at the beginning of the 14-day experimental period would overestimate the rate of oxidation, while those obtained at the end of the experimental period would not. This would cause the slope of the regression line to misrepresent the actual effect of the diet on the metabolism of the bird. However, since there was no data collected on the metabolic rate of the birds during the experiment, the data cannot be adjusted for changes in metabolism.

For these reasons, the experiment was repeated with eight birds (four males and four females), with adjustments made to ensure the birds were familiar with the environmental and experimental conditions. Table 4.10 shows the regression equations, R^2, P values and availability of raw peas, cooked peas and cooked peas plus lysine for Experiment 3. The regression equations were significant ($P<0.05$) for each of the eight birds. R^2 values ranged from 0.9163 to 0.9939. Table 4.8 shows the average availabilities for male, female and all birds combined. There were no statistical differences in availability, slope or intercept of the regression lines for raw peas, cooked peas or peas plus lysine between male and female birds. Therefore the data for all birds combined was used for comparison with the slope ratio data. The average availability of raw peas was shown to be 61.8 ± 3.48%, with 95% confidence interval of 53.6 – 70.1%. Although a statistical comparison between the IAAO method values and the slope ratio values was not possible, due to the nature of the slope ratio calculations, the values from the slope ratio trial fell within the 95% confidence interval from the IAAO method, with the exception of lysine availability in males using feed conversion as the response variable. These values, however, were very close to the upper 95% confidence interval. The availability values calculated using both male and female broilers (57.1% and 64.3% using lysine accretion and feed conversion data respectively) both fell within the 95% confidence interval for lysine availability in raw peas found in Experiment 3.

The availability values obtained for the cooked peas showed that the availability of lysine was reduced from the initial value of 61.8% to an average of 34.8 ± 6.74%. Variation in the availability of the cooked peas was higher than that of raw peas, suggesting that individual birds may utilize heat-damaged proteins at different efficiencies. There has previously been no report of this, as the standard slope-ratio

technique requires the response variable to be averaged over several animals. Adding purified lysine back to the cooked pea diet resulted in an increase in the availability of lysine to 93.6 ± 8.80%. The variation seen here is likely due to variation in the lysine requirement of individual birds and in the individual birds ability to utilize heat-damaged protein. The amount of lysine that was added back to the diet was calculated to bring the availability up to 100%, assuming the lysine in the peas was rendered completely unavailable. Since there was some available lysine in the cooked peas, values of greater than 100% were obtained for some individual birds (Table 4.10). The availability of lysine in the cooked peas plus lysine-HCl diet would be influenced by the requirement of each bird for lysine. Once the requirement was met, oxidation of L-[1-^{14}C]phenylalanine would no longer increase with additional available lysine. The diets were formulated to be 15% below the average lysine requirement for broilers, indicating that remaining available lysine in the peas that caused the diet to exceed this requirement would result in underestimation of available lysine. The use of this method for determination of feedstuff lysine availability would not be subject to this effect, as only diets deficient in total lysine would be used.

The pattern of oxidation of the indicator shown in Figure 4.5 shows an example bird from Experiment 3. The response line labeled pure lysine represents the response in indicator oxidation to lysine that is assumed to be 100% available. Since it is 100% available, all other response lines should have a slope that is less than that of the pure lysine line. The line representing raw peas has a smaller slope, indicating an availability of lysine less than that of lysine-HCl. In this case, the availability of lysine in raw peas was 58.0%. The line representing cooked peas has a slope less than both the raw peas and the purified lysine curves, showing that the availability was reduced through cooking. The availability was calculated to be 41.1%. Since the diet with cooked peas and pure

lysine was formulated assuming the availability of lysine in cooked peas would be zero, the slope of that line was greater than the slope of the pure lysine line. The availability of lysine in that diet was 79.4%.

The results of Experiment 3 show that the IAAO method can be used to determine the metabolic availability of amino acids in feedstuffs. The values obtained for peas were similar to those obtained by the more traditional slope ratio assay. Experiment 2 showed that the conditions of the birds in the experiment must be controlled. The two birds that did not respond to the dietary levels as predicted were thought to have been affected by the conditions of the experiment. The novel environment and noise generated by the vacuum pump during the experiment may have lead to an increase in stress and consequently, metabolic rate that would alter the proportion of liberated $^{14}CO_2$ that was exhaled during the collection period. Since metabolic rate was not measured, and there is currently no method available to compensate for changes in metabolic rate, birds must be allowed to adjust to the experimental conditions. This method for determining the metabolic availability of essential amino acids in poultry provides a rapid method to evaluate the feeding value of feedstuffs. It also provides information on the between-bird variation in the ability to digest and utilize amino acids, and allows calculation of confidence intervals.

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Table 4.1. Dietary ingredients and calculated nutrient composition of diets for the slope ratio experiment (Experiment 1) determining the availability of lysine in a sample of peas

Ingredient:	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
				%			
Barley	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Wheat Shorts	13.50	13.50	13.50	13.50	13.50	13.50	13.50
Wheat	15.45	15.45	15.45	15.45	15.45	15.45	15.45
Feather Meal	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Corn Oil	10.00	10.50	11.00	12.00	12.00	12.00	12.00
Soybean Meal	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Canola Meal	7.00	7.00	7.00	7.00	7.00	7.00	7.00
Corn Gluten Meal	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Corn Distillers Grain	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Vitamin/Mineral Premix[1]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Dical Phos	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Limestone	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Salt	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Amino Acid Mixture[2]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Glutamate	7.50	6.00	4.00	0.00	7.40	7.30	7.10
Corn Starch	18.85	13.35	6.35	1.35	16.81	16.77	16.84
Peas	0	6.50	15.00	23.00	0	0	0
Lysine- HCl	0	0	0	0	0.14	0.28	0.41
Calculated Analysis							
ME (kcal/kg)	3157	3154	3148	3157	3156	3156	3156
CP	24.61	24.66	24.68	24.58	24.65	24.69	24.62
Ca	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Available P	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Lysine (calculated)	0.55	0.65	0.75	0.85	0.65	0.75	0.85
Lysine (analyzed)	0.65	0.69	0.80	0.92	0.71	0.78	0.92

[1] The vitamin mineral premix provided (per kg of feed): vitamin A, 10,000 IU; vitamin D_3, 2,500 IU; vitamin K, 2.0 mg; pantothenic acid, 14 mg; riboflavin, 5.0 mg; folacin, 0.80 mg; biotin, 1.8 mg; niacin, 65 mg; thiamine, 2.0 mg; pyridoxine, 4.0 mg; vitamin B_{12}, 0.015 mg; Vitamin E, 35 IU; Mn, 70 mg; Cu, 8.5 mg; Zn, 80 mg; Se, 0.10 mg; Fe, 100 mg; choline, 1,000 mg.

[2] The amino acid mixture provided (per kg of feed): methionine, 0.3%; threonine, 0.2%; Tyrosine, 0.17%; Arginine, 0.025%; Histidine, 0.005%; Cystine, 0.005%; Isoleucine, 0.015%; Valine, 0.009%.

Table 4.2: Dietary amino acid composition of diets for the slope ratio experiment (Experiment 1) determining the availability of lysine in a sample of peas, as determined by HPLC

Amino Acid (%)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Serine	0.952	0.642	0.842	0.865	0.639	0.871	0.875
Histidine	0.346	0.321	0.397	0.347	0.322	0.324	0.357
Glysine	1.252	1.053	1.494	1.400	1.155	1.063	2.575
Threonine	0.794	0.727	0.766	0.786	0.770	0.723	0.766
Arginine	1.125	1.299	1.232	1.211	1.168	1.280	1.187
Alanine	0.928	0.760	0.981	0.981	0.827	0.908	1.330
Tyrosine	0.735	0.675	0.752	0.680	0.764	0.728	0.696
Methionine	0.322	0.321	0.345	0.346	0.430	0.397	0.399
Valine	1.030	0.920	1.032	0.942	0.840	0.970	1.022
Phenylalanine	0.782	0.791	0.809	0.778	0.783	0.792	0.796
Isoleucine	0.770	0.757	0.726	0.756	0.786	0.793	0.735
Leucine	1.278	1.174	1.351	1.390	1.138	1.226	1.172
Lysine	0.550	0.531	0.747	0.826	0.570	0.664	0.695
Aspartic Acid	1.238	0.927	1.316	1.346	1.000	1.402	1.364
Glutamic Acid	3.388	2.988	3.631	3.578	2.983	3.398	3.509

Table 4.3. Dietary treatments for the indicator amino acid oxidation experiment (Experiment 2 and 3) designed to determine the availability of lysine in a sample of peas

Ingredient:	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
				%			
Wheat	14.40	14.40	14.40	14.40	14.40	14.40	14.40
Barley	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Wheat Shorts	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Corn Gluten Meal	12.00	12.00	12.00	12.00	12.00	12.00	12.00
Canola Meal	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Soybean Meal	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Feather Meal	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Corn Distillers Grain	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Corn Oil	10.00	10.00	10.00	10.00	14.27	14.27	14.27
Vitamin Mineral Premix[1]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Limestone	2.10	2.10	2.10	2.10	2.10	2.10	2.10
Dical Phos	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Salt	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Amino Acid Mixture[2]	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Corn Starch	21.17	21.14	21.17	21.17	0	0	0
Glutamate	3.07	2.94	2.81	2.70	0.37	0.37	0.00
Phenylalanine	0.16	0.16	0.16	0.16	0.00	0.00	0.00
Lysine HCl	0.00	0.16	0.26	0.37	0.00	0.00	0.37
Peas	0.00	0.00	0.00	0.00	20.00	20.00	20.00
Calculated Analysis:							
ME (kcal/kg)	3408	3403	3398	3394	3401	3401	3402
CP	24.7	24.7	24.7	24.7	24.7	24.7	24.7
Ca	1.01	1.01	1.01	1.01	1.01	1.01	1.01
Available P	0.46	0.46	0.46	0.46	0.46	0.46	0.46
Lysine (calculated)	0.55	0.65	0.75	0.85	0.85	0.85	1.25
Lysine (analyzed	0.58	0.62	0.71	0.83	0.83	0.83	1.26
Lysine (analyzed	0.57	0.69	0.73	0.83	0.88	0.80	1.24

[1]The vitamin mineral premix provided the following (per kg of feed): vitamin A, 10,000 IU; vitamin D_3, 2,500 IU; vitamin K, 2.0 mg; pantothenic acid, 14 mg; riboflavin, 5.0 mg; folacin, 0.80 mg; biotin, 1.8 mg; niacin, 65 mg; thiamine, 2.0 mg; pyridoxine, 4.0 mg; vitamin B_{12}, 0.015 mg; vitamin E, 35 IU; Mn, 70 mg; Cu, 8.5 mg; Zn, 80 mg; Se, 0.10 mg; Fe, 100 mg; choline, 1,000 mg.

[2]The amino acid mixture provided the following (per kg of feed): Methionine, 0.26; Histidine, 0.15; Threonine, 0.375; Arginine, 0.375; Tyrosine, 0.225; Isoleucine, 0.225; Valine, 0.15.

Table 4.4. Dietary treatments for the indicator amino acid oxidation experiment (Experiment 2) designed to determine the availability of lysine in a sample of peas

Amino Acid (%)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Serine	0.773	0.710	0.773	0.977	0.752	0.695	0.750
Histidine	0.346	0.352	0.384	0.331	0.330	0.329	0.332
Glysine	1.098	1.140	1.219	1.345	1.276	1.140	1.248
Threonine	0.885	0.804	0.805	0.797	0.803	0.793	0.801
Arginine	1.219	1.276	1.324	1.247	1.213	1.339	1.384
Alanine	0.809	0.861	0.880	0.909	0.888	0.792	0.849
Tyrosine	0.699	0.706	0.710	0.662	0.675	0.698	0.661
Methionine	0.304	0.303	0.271	0.338	0.271	0.317	0.294
Valine	0.910	0.947	0.868	1.045	0.885	0.821	0.861
Phenylalanine	0.732	0.753	0.742	0.803	0.783	0.748	0.761
Isoleucine	0.736	0.745	0.799	0.726	0.721	0.787	0.697
Leucine	1.160	1.216	1.116	1.333	1.172	1.079	1.120
Lysine	0.585	0.612	0.621	0.784	0.615	0.557	0.522
Aspartic Acid	1.067	1.040	1.090	1.473	1.081	0.995	0.962
Glutamic Acid	3.143	3.155	3.245	3.966	3.274	3.055	3.143

Table 4.5. Dietary treatments for the indicator amino acid oxidation experiment (Experiment 3) designed to determine the availability of lysine in a sample of peas

Amino Acid (%)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Serine	0.772	0.716	0.775	0.987	0.753	0.759	0.755
Histidine	0.456	0.454	0.483	0.430	0.436	0.430	0.438
Glysine	1.103	1.145	1.222	1.358	1.288	1.145	1.254
Threonine	0.794	0.802	0.715	0.816	0.800	0.802	0.812
Arginine	1.182	1.189	1.226	1.254	1.217	1.144	1.186
Alanine	0.811	0.863	0.884	0.911	0.893	0.796	0.858
Tyrosine	0.602	0.614	0.713	0.664	0.580	0.602	0.667
Methionine	0.307	0.306	0.271	0.346	0.275	0.325	0.303
Valine	0.919	0.953	0.872	1.053	0.896	0.824	0.865
Phenylalanine	0.733	0.758	0.755	0.734	0.758	0.753	0.752
Isoleucine	0.745	0.756	0.808	0.838	0.823	0.798	0.804
Leucine	1.167	1.223	1.160	1.336	1.174	1.186	1.126
Lysine	0.573	0.691	0.738	0.837	0.886	0.804	1.248
Aspartic Acid	1.075	1.040	1.095	1.473	1.872	1.007	0.983
Glutamic Acid	3.141	3.165	3.253	3.971	3.271	3.065	3.142

Table 4.6. Total BW gain, feed conversion (g feed/g gain) and lysine accretion for birds from 20 to 30 days of age fed diets for the slope ratio assay for lysine (Experiment 1)

Diet:[3]	Lysine Dose Level (g Lys/kg Feed):[1]	Male		Female	
		Form of Lysine Addition			
		Free Lysine	Peas	Free Lysine	Peas
		Live Weight Gain (g)			
1	0	241.1	------	264.8	------
2/5	0.4	277.6	260.7	291.2	248.7
3/6	1.5/1.3[2]	285.0	267.9	313.9	271.0
4/7	2.7	363.2	268.8	364.4	264.3
		SEM = 18.95		SEM = 17.45	
		Feed Conversion (g feed/g gain)			
1	0	2.963	------	2.78	------
2/5	0.4	2.593	2.838	2.46	2.64
3/6	1.5/1.3	2.468	2.663	2.21	2.51
4/7	2.7	1.995	2.363	1.99	2.30
		SEM = 0.1618		SEM = 0.110	
		Lysine Accretion (g)			
1	0	0.965	------	1.125	------
2/5	0.4	1.150	0.878	1.733	1.933
3/6	1.5/1.3	1.715	1.333	3.053	2.368
4/7	2.7	2.110	1.688	3.435	2.650
		SEM = 0.2615		SEM = 0.3020	

[1] Lysine increment is the amount of lysine (g/kg) above that of the basal diet, which contained 6.5 g lysine per kg diet.

[2] When two numbers are present, the first refers to the diet containing purified lysine, and the second to the diet containing peas as a source of lysine.

[3] Diets are those described in Table 4.1. Diets 1 - 4 contain free lysine at levels of 6.5, 6.9, 8.0, and 9.2 g lysine per kg diet respectively. Diets 5-7 were formulated by adding raw ground peas to Diet 1 (basal diet) at the expense of cornstarch, and contain 7.1, 7.8 and 9.2 g lysine per kg diet respectively.

Table 4.7. Linear regression data and availability values for live weight gain, feed conversion, and lysine accretion of 20 to 30 day old broilers fed diets for a slope ratio assay for lysine (Experiment 1)

Source of Lysine	Gain (g) Equation	R^2	P value	Feed Conversion (kg/kg) Equation	R^2	P value	Lysine Accretion (g) Equation	R^2	P value
Male[3]									
Free Lysine	y = 39.0x + 245.9	0.73	0.0001	y = -0.306x + 2.86	0.76	0.0001	y = 0.415x + 1.00	0.45	0.0042
Peas	y = 8.5x + 249.7	0.05	0.4011	y = -0.215x + 2.96	0.30	0.0276	y = 0.291x + 0.87	0.32	0.0220
Availability (%)[1]	N/A[2]			70.3			70.1		
Female[3]									
Free Lysine	y = 34.7x + 268.8	0.71	0.0001	y = -0.272x + 2.67	0.56	0.0014	y = 0.892x + 1.34	0.73	0.0001
Peas	y = 2.1x + 259.7	0.01	0.6727	y = -0.167x + 2.75	0.43	0.0060	y = 0.497x + 1.44	0.47	0.0034
Availability (%)[1]	N/A[2]			61.4			55.7		

[1] Availability was calculated by taking the slope of the line derived from adding peas to the diet as a percentage of the line derived by adding free lysine to the diet.

[2] Availability was not calculated if one or both of the linear regression equations was not significant ($P < 0.05$).

[3] Regression equations were based on data obtained on four bird per gender per diet, totaling 28 male and 28 female birds. The data obtained for the basal diet was used in both the free lysine and peas regression equations.

Table 4.8. Percent ^{14}C-phenylalanine dose oxidized values for broilers fed availability diets including a basal diet (Diet 1), and incremental increases in lysine by adding free lysine (Diets 2-4), raw peas (Diet 5), cooked peas (Diet 6), and cooked peas plus lysine (Diet 7) as described in Table 2 (Experiment 2)

Diet	Lysine Increment (g/kg)[1]	Bird 1 (% dose oxidized)	Bird 2 (% dose oxidized)	Bird 3 (% dose oxidized)	Bird 4 (% dose oxidized)
1	0	16.83	8.67	23.81	26.45
2	0.4	14.43	9.78	22.97	26.06
3	1.3	16.90	9.62	21.24	22.55
4	2.5	11.99	10.61	17.92	19.30
5	2.5	21.99	13.04	19.19	21.20
6	2.5	17.34	9.52	23.27	22.90
7	4.8	22.69	11.19	15.83	19.07

[1] **Lysine increment is the amount of lysine (g/kg) above that of the basal diet, which contained 5.5 g lysine per kg diet, based on analyzed values in Table 4.2.**

Table 4.9. Linear regression equations, R^2, P values and availability values determined using 19 to 32 day old male broilers using the indicator amino acid oxidation procedure (Experiment 2)

	Regression Equation	R^2	P value	Availability
Bird 1:				
Free Lysine	y = -0.121x + 16.85	0.44	0.3334	
Raw Peas[1]	y = 0.172x + 16.83			N/A[2]
Cooked Peas[1]	y = 0.017x + 16.83			N/A
Cooked Peas + Lys[1]	y = 0.195x + 16.83			N/A
Bird 2:				
Free Lysine	y = 0.056x + 8.82	0.84	0.0813	
Raw Peas	y = 0.145x + 8.67			N/A
Cooked Peas	y = 0.082x + 8.67			N/A
Cooked Peas + Lys	y = 0.028x + 8.67			N/A
Bird 3:				
Free Lysine	y = -0.194x + 24.39	0.92	0.0390	
Raw Peas	y = -0.154x + 23.81			79.4
Cooked Peas	y = -0.018x + 23.81			9.3
Cooked Peas + Lys	y = -0.266x + 23.81			137.1
Bird 4:				
Free Lysine	y = -0.250x + 27.33	0.92	0.0394	
Raw Peas	y = -0.175x + 26.45			70.0
Cooked Peas	y = -0.1186x + 26.45			47.5
Cooked Peas + Lys	y = -0.2462x + 26.45			98.4

[1] Regression equations for the raw peas, cooked peas, and cooked peas plus supplemental lysine were determined using only two points. These were the unsupplemented basal diet (diet 1) and the test diet for that treatment. Linearity for oxidation rate with increased lysine in other forms was assumed when linearity was achieved with free lysine. Therefore no P value or R^2 where presented.

[2] Availability was not calculated when linearity was not significant for the diets supplemented with free lysine.

Table 4.10. Linear regression equations, R^2, P values and availability values determined using 19 to 32 day old male and female broilers using the indicator amino acid oxidation procedure (Experiment 3)

	Regression Equation	R^2	P value	Availability		Regression Equation	R^2	P value	Availability
	----------Males----------					----------Females----------			
Bird 1:					Bird 1:				
Free Lysine	y = -0.379x + 22.39	0.9163	0.0427		Free Lysine	y = -0.161x + 21.95	0.9318	0.0347	
Raw Peas[1]	y = -0.220x + 22.77			58.0	Raw Peas[1]	y = -0.086x + 22.00			53.2
Cooked Peas[1]	y = -0.156x + 22.77			41.1	Cooked Peas[1]	y = -0.079x + 22.00			49.1
Cooked Peas + Lys[1]	y = -0.301x + 22.77			79.4	Cooked Peas + Lys[1]	y = -0.249x + 22.00			154.7
Bird 2:					Bird 2:				
Free Lysine	y = -0.218x + 25.65	0.9939	0.0031		Free Lysine	y = -0.257x + 24.85	0.9883	0.0059	
Raw Peas	y = -0.162x + 25.50			74.3	Raw Peas	y = -0.123x + 24.74			47.9
Cooked Peas	y = -0.106x + 25.50			48.6	Cooked Peas	y = -0.106x + 24.74			41.2
Cooked Peas + Lys	y = -0.193x + 25.50			88.5	Cooked Peas + Lys	y = -0.205x + 24.74			79.8
Bird 3:					Bird 3:				
Free Lysine	y = -0.288x + 25.65	0.9326	0.0343		Free Lysine	y = -0.296x + 18.22	0.9743	0.0129	
Raw Peas	y = -0.194x + 25.00			67.4	Raw Peas	y = -0.219x + 17.79			70.9
Cooked Peas	y = -0.143x + 25.00			49.7	Cooked Peas	y = -0.027x + 17.79			9.1
Cooked Peas + Lys	y = -0.248x + 25.00			86.1	Cooked Peas + Lys	y = -0.250x + 17.79			84.4
Bird 4:					Bird 4:				
Free Lysine	y = -0.161x + 25.17	0.9473	0.0267		Free Lysine	y = -0.209x + 28.10	0.9443	0.0282	
Raw Peas	y = -0.086x + 24.89			53.4	Raw Peas	y = -0.145x + 27.84			69.4
Cooked Peas	y = -0.001x + 24.89			0.60	Cooked Peas	y = -0.081x + 27.84			38.8
Cooked Peas + Lys	y = -0.148x + 24.89			91.9	Cooked Peas + Lys	y = -0.176x + 27.84			84.2

[1] Regression equations for the raw peas, cooked peas, and cooked peas plus supplemental lysine were determined using only two points. These were the unsupplemented basal diet (diet 1) and the test diet for that treatment. Linearity for oxidation rate with increased lysine in other forms was assumed when linearity was achieved with free lysine. Therefore no P value or R^2 where presented.

[2] Availability was not calculated when linearity was not significant for the diets supplemented with free lysine.

Table 4.11. Availability values, SEM and 95% confidence intervals for male, female and all birds as determined using the IAAO technique (Experiment 3)

	Lysine Availability (%)	Standard Error	95% CI (%)
Males (n=4)			
Raw Peas	63.4	5.25	47.5 – 73.2
Cooked Peas	35.0	10.29	9.4 – 59.7
Cooked Peas + Lys	86.5	12.90	55.0 – 118.0
Females (n=4)			
Raw Peas	60.4	5.25	47.5 – 73.2
Cooked Peas	34.6	10.29	9.4 – 59.7
Cooked Peas + Lys	100.8	12.90	69.3 – 132.3
All Birds (n=8)			
Raw Peas	61.8	3.48	53.6 – 70.1
Cooked Peas	34.8	6.74	18.8 – 50.7
Cooked Peas + Lys	93.6	8.8	72.7 – 114.5

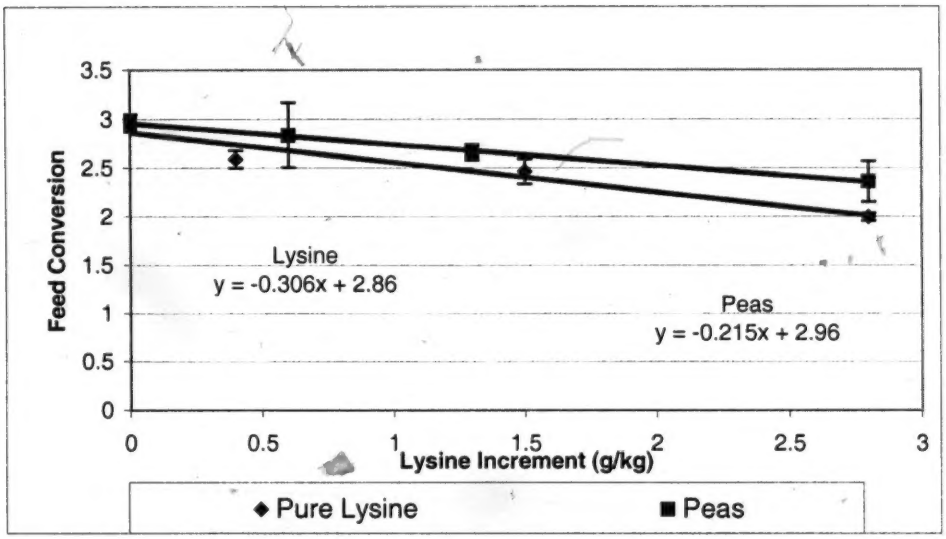

Figure 4.1. Lysine availability in raw peas as determined by a slope ratio experiment using feed conversion as the response criteria in male broilers between 20 and 30 days of age (Experiment 1). Seven diets were fed, a basal diet containing 6.5 g lysine per kg diet, three diets with incremental increases in lysine as lysine-HCl, and three diets with incremental increases in lysine in the form of peas. Each diet was fed to 4male broiler. Response criteria were plotted versus the lysine increment and the slopes of the response curves were compared to determine the availability of lysine in peas. The availability of lysine was (-0.215/-0.306)*100 = 70.3%.

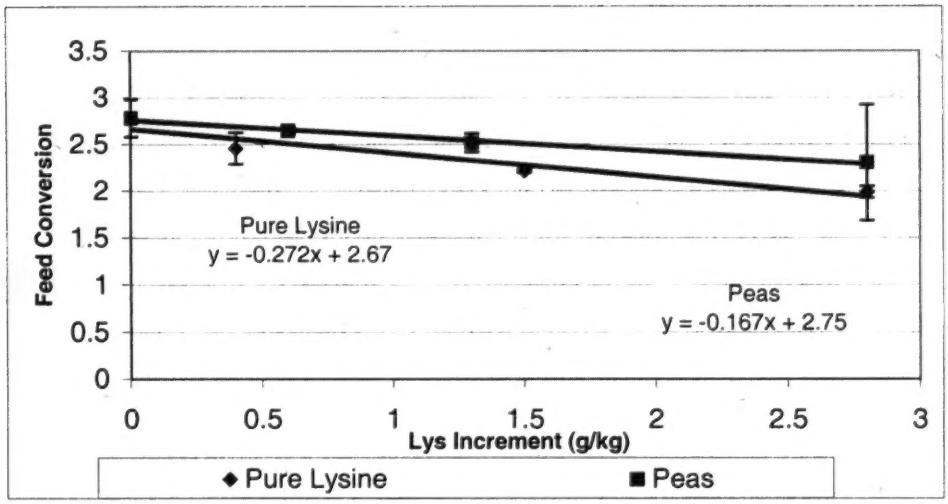

Figure 4.2. Availability of lysine in raw peas as determined by a slope ratio experiment using feed conversion as the response criteria in female broilers between 20 and 30 days of age (Experiment 1). Seven diets were fed, a basal diet containing 6.5 g lysine per kg diet, three diets with incremental increases in lysine as lysine-HCl, and three diets with incremental increases in lysine in the form of peas. Each diet was fed to 4 female broilers. Response criteria were plotted versus the lysine increment and the slopes of the response curves were compared to determine the availability of lysine in peas. The availability of lysine was (-0.167/-0.272)*100 = 61.4%.

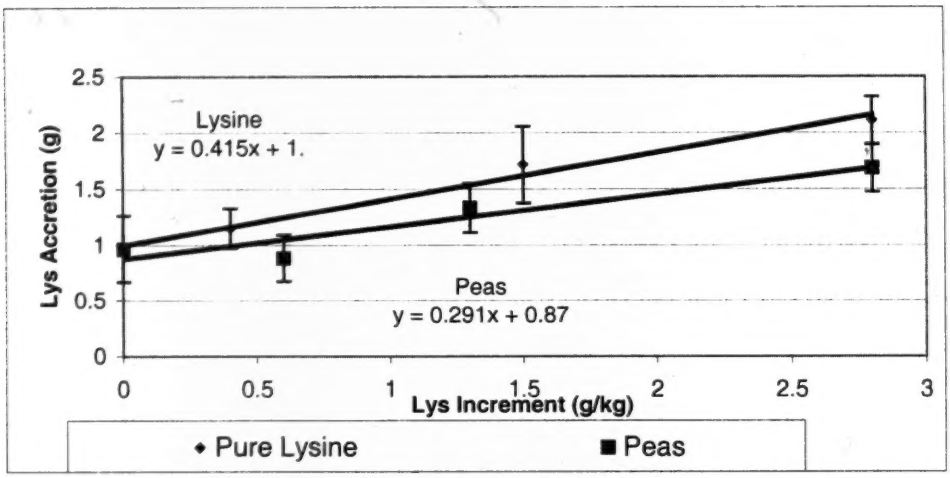

Figure 4.3. Availability of lysine in raw peas as determined using a slope ratio experiment with lysine accretion as the response criteria in male broilers between 20 and 30 days of age (Experiment 1). Seven diets were fed, a basal diet containing 6.5 g lysine per kg diet, three diets with incremental increases in lysine as lysine-HCl, and three diets with incremental increases in lysine in the form of peas. Each diet was fed to 4 male broilers. Response criteria were plotted versus the lysine increment and the slopes of the response curves were compared to determine the availability of lysine in peas. The availability of lysine in raw peas was (0.291/0.415)*100 = 70.1%.

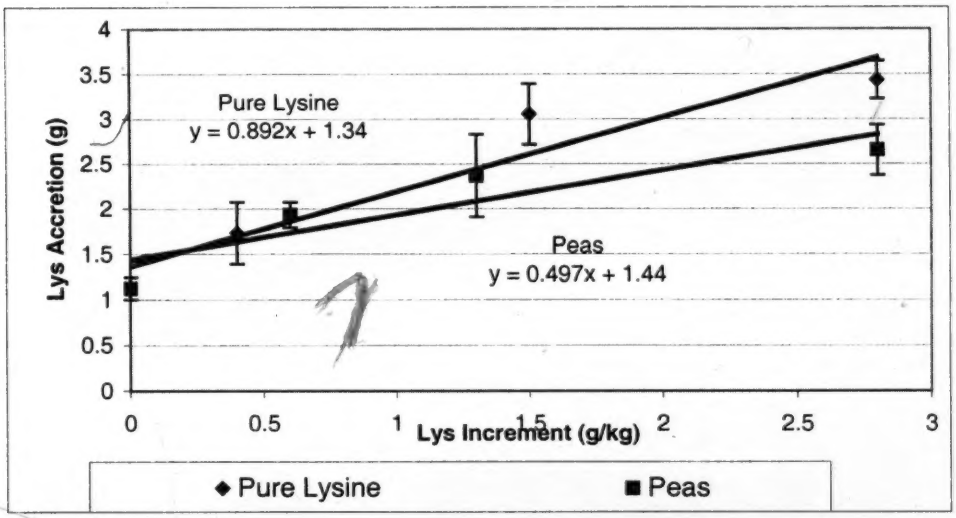

Figure 4.4. Availability of lysine in raw peas as determined using a slope ratio experiment with lysine accretion as the response criteria in female broilers between 20 and 30 days of age (Experiment 1). Seven diets were fed, a basal diet containing 6.5 g lysine per kg diet, three diets with incremental increases in lysine as lysine-HCl, and three diets with incremental increases in lysine in the form of peas. Each diet was fed to 4 female broilers. Response criteria were plotted versus the lysine increment and the slopes of the response curves were compared to determine the availability of lysine in peas. The availability of lysine in the peas was (0.497/0.892)*100 = 55.7%.

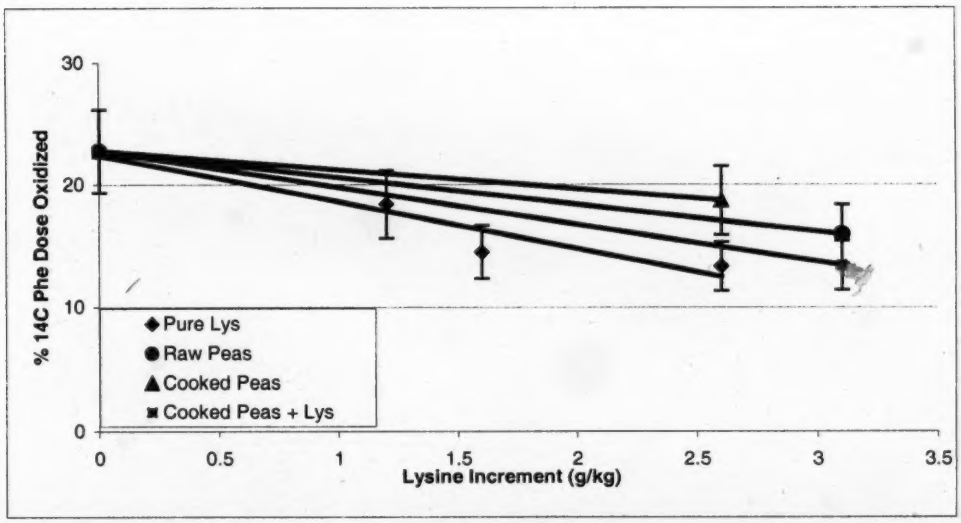

Figure 4.5: The % L-[1-^{14}C]phenylalanine dose oxidized for diets differing in lysine content and source of lysine. The response curve to increments of crystalline lysine is represented by the equation y = -0.379x + 22.39. The equations representing the response to raw peas, cooked peas and cooked peas plus supplemental lysine are y = -0.220x + 22.77, y = -0.156x + 22.77, and y = -0.301x + 22.77 respectively. The initial response curve was generated using four diets (diets 1 through 4 from Table 4.2), while the remaining three lines were generated using only the basal diet (diet 1) and either diet 5, 6, or 7 for raw peas, cooked peas, or cooked peas plus lysine respectively. It was assumed that if added increments of pure lysine resulted in a linear decrease in the oxidation of L-[1-^{14}C]phenylalanine, the response to lysine in the form of the test ingredient would also be linear. Error bars were added using the SEM associated with the plateau oxidation of the indicator for each diet.

CHAPTER 5: CONCLUSION

A major source of inefficiency in the poultry industry is the formulation of rations on the basis of total amino acid values for the requirements of chickens and the feeding value of feedstuffs (NRC, 1994). There are several methods available to determine the digestibility of amino acids in feedstuffs, and the swine industry has adopted the ileal digestibility method for formulation of swine diets (NRC, 1998). Digestibility estimates are an improvement over the use of total amino acid values, as they account for amino acids that are not absorbed by the animal (Sibbald, 1987). Availability estimates, however, are preferable as they also account for amino acids that are absorbed in a form that is not metabolically active (Sibbald, 1987; van Barneveld et al., 1994). Currently, methods that can be used to determine the availability of an essential amino acid are time consuming, labour intensive and costly. The experiments presented here were designed to develop a new, rapid method for determining the metabolic availability of dietary indispensible amino acids in feedstuffs for poultry.

Chapter 1 described the determination of the bicarbonate retention factor (BRF) for broilers, weekly from hatch to 42 days of age. Since the collection of liberated CO_2 is incomplete during a practical timeframe, a BRF is necessary to compensate for the loss of CO_2 in slowly exchanging pools and metabolic products. The BRF was determined using a primed oral dosing protocol designed to approximate a constant infusion. $Na^{14}CO_3$ was used as the tracer, and the percent of the tracer retained by the bird was determined at the plateau in excreted $^{14}CO_2$. The results for day-of-hatch chicks were considered to be unusable, due to the high degree of variation between the birds. This was thought to be due to variable water consumption among birds, although this was not measured in the

experiment. The data for the remaining days were used to develop a prediction equation using BW to predict bicarbonate retention. The equation $y = -5.5391*10^{-12}X^4 + 5.318986*10^{-8}X^3 - 1.5899*10^{-4}X^2 + 0.1587X + 13.3228$ describes the relationship between BW (X) and bicarbonate retention (Y) ($R^2 = 0.8882$).

Whereas bicarbonate retention experiments are currently the most accurate method of compensating for irreversible loss of labelled CO_2 during substrate oxidation studies, there are criticisms of this approach. It is thought that, since the site of appearance of the $^{14}CO_2$ in retention studies is the plasma pool, and the site of appearance of bicarbonate in oxidation studies is the intracellular pool, retention studies may not accurately represent the irreversible loss of bicarbonate (Hamel et al., 1993). Specific studies have shown both that the infused bicarbonate fails to reach equilibrium with the intracellular pools (Hamel et al., 1993) and that the infusion protocols do in fact accurately represent the re-incorporation of bicarbonate (Tomera et al., 1983). Despite these conflicting results, it seems intuitive that the bicarbonate in the plasma pool would not equilibrate with the intracellular pool, and since the latter is the site of CO_2 production it would necessarily show a net loss of CO_2. This would suggest that, even with the use of bicarbonate retention factors, substrate oxidation studies would underestimate the actual rate of oxidation of the substrate. With this in mind, the bicarbonate retention factor is currently the most accurate method available to compensate for the incomplete collection of labelled CO_2.

Chapter 2 described two studies necessary for the application of the IAAO method to broilers. Experiment 1 was designed to determine the appropriate priming and hourly oral dose of L-[1-^{14}C]phenylalanine. Different combinations of priming and oral dose were tested to determine the combination that resulted in the most rapid attainment

of isotopic steady state. The combination of prime and oral dose that resulted in the most rapid achievement of exhaled $^{14}CO_2$ plateau was found to be 3 μCi/kg BW (prime) and 3.5 μCi/kg BW/h (hourly oral dose), representing a ratio of 0.87 (prime: oral dose). All birds that were given this dose achieved plateau within the 4-hour oxidation study. It took 30 to 120 min to achieve plateau, leaving 120 to 210 minutes to determine to average oxidation rate. In order to calculate the plateau value, a minimum of three points must be available on the curve, so steady state must be reached within 150 minutes. The rate of oxidation is more accurately estimated using a greater number of data points on the plateau. The use of a prime and oral dose protocol will provide four to seven data points with which to calculate the percentage of the infused dose which was oxidized.

The IAAO method involves the feeding of multiple levels of a test amino acid. Experiment 2 was designed to determine the period of time required for the bird to reach a new and stable rate of oxidation of the indicator amino acid (^{14}C-phe) when the dietary concentration of the test amino acid was changed. The results showed that one-day was sufficient when the diet was changed from a deficient to a sufficient level of lysine, whereas two days were required when the diet was changed from a sufficient to a deficient level of lysine. These results were in agreement with studies in swine (Ball et al., 2002) and in humans (Thorpe et al., 1999). Hoerr et al. (1993) showed that feeding a low protein diet to humans resulted in a decrease in protein breakdown as well as synthesis. Upon consumption of a high protein diet, protein synthesis increased whereas breakdown was further reduced. This was explained as a carry-over effect designed to conserve amino acids. This conservation response could help explain the need for a two-day adaptation period when changing from a sufficient to a deficient level of lysine found in Chapter 2.

The experiments presented in Chapter 4 were designed to test the IAAO method for use in a slope ratio-type trial to determine the availability of lysine in poultry feedstuffs. Peas were used, not because they are a common ingredient in poultry feed, but because the represent a relatively high protein feedstuff (about 20% CP) that is not commonly heated during processing. This allowed testing of the ability of the IAAO method to account for the metabolic unavailability of products of the Maillard reaction. The availability values determined by the traditional slope ratio trial were similar to those determined by IAAO method. The slope ratio availabilities for female birds (55.7%) and for male birds (70.1%) using lysine accretion as the response variable were within the 95% confidence interval for the IAAO method, whereas the values obtained for male birds using feed consumption as the response variable (70.3%) were not. This value, however, was very close to the 95% confidence interval (IAAO method 95% CI = 53.6-70.1%). The availability values obtained by pooling the male and female data were 57.1% using lysine accretion data and 64.3% using feed conversion, both of which were within the confidence intervals determined in the IAAO method trial. In Experiment 3 of Chapter 4, the peas were heated to 165 C for 4 hours in order to cause a reduction in the availability of lysine (van Barneveld et al., 1994). According to the IAAO method, the availability was reduced from 64.8% in raw peas to 34.8% for cooked peas. It was confirmed that the decreased availability of lysine was the cause of the increased oxidation of the indicator by adding lysine-HCl back into the cooked pea diet. This resulted in an increase in the oxidation of L-[1-^{14}C]phenylalanine with some individual bird values above 100%. These results show that for broilers, the IAAO technique can be used in a slope ratio type trial in order to determine the availability of essential amino acids in feedstuffs. It also shows that the method is sensitive to changes in lysine availability due to heating.

Further advantages of this method are that it is are rapid, and provides information of the between bird variation in availability. This variation cannot be studied using the slope ratio method, and may help researchers better understand how to efficiently feed poultry.

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